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Selective Endothelin Receptor Involvement in the Development of Colorectal Cancer and Liver Metastases

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STATEMENT OF ORIGINALITY

The studies described and presented in this thesis are the original work of the author. All work was performed by the author with the following exceptions:

- All autoradiography was performed in conjunction with Dr Michael Dashwood in Clinical Biochemistry, The Royal Free and University College Medical School, London.
- All flow cytometry was performed in conjunction with Dr Derek Davies at the Imperial Cancer Research Fund (Cancer Research UK).
- All specimens of colorectal liver metastases were collected, with patient consent, from surgical resections carried out by Professor I Taylor at The Middlesex Hospital, London.

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university, or other institute of learning.

All animal studies were performed under appropriate Home Office project and personal licences.

CONTENTS

Abstract

Selective Endothelin Receptor Involvement in the Development of Colorectal Cancer and Liver Metastases

Endothelin-1 (ET-1), a vasoactive peptide with mitogenic qualities, is over-expressed in the plasma of patients with colorectal cancer (CRC) and liver metastases. Its actions are mediated via two G-protein linked receptors, ET_A and ET_B. This study (i) examined the effect of ET-1 and its receptor antagonists as well as a G-protein blocker on CRC cell line growth; (ii) investigated tET receptor distribution in human CRC and liver metastases by autoradiography; (iii) recorded the effect on tumour growth of an oral ET_A receptor antagonist to an in vivo model of colorectal cancer liver metastases. ET-1 stimulated significant growth in the cancer cells. This effect was reversed by the ET_A, but not the ET_B, receptor antagonist. Apoptosis was similar in controls and ET-1± antagonist treated cells. BrDU staining demonstrated an ET-1 dependent increase in mitosis, reversed by the ET_A receptor antagonist. Blocking the G-protein subunits also reversed growth. In human cancer tissues ET_A was over-expressed while ET_B was under-expressed compared to controls. There was no significant difference in weight or number of liver metastases between control and experimental rats in vivo. In summary, ET-1 stimulates human CRC growth in vitro via ET_A receptors by mechanisms that include stimulation of mitosis but not alteration of apoptosis. Thi signal is transduced via the G-protein subunits Go or Gi. Human CRC liver metastases tissue over-express ET_A receptors compared to normal tissue. ET receptor antagonists may have a therapeutic role in primary and metastatic CRC.

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List of Abbreviations

5-FU	5-flurouracil
AJCC	The American Joint Committee on Cancer
APC	adenomatous polyposis coli
BrDU	5-bromo-2'-deoxyuridine
CEA	Carcinoembryonic antigen
COX-1/COX-2	Cyclo-oxygenase-1 /-2
CRC	Colorectal cancer
CT	Computerised tomography
DAB	3,3'-diaminobenzidine tetrahydrochloride
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ET	Endothelin
ET-1/ET-2/ET-3	Endothelin-1 / -2 / -3
FAP	Familial adenomatous polyposis
FCS	Foetal calf serum
FDG-PET	F-18 fluorodeoxyglucose PET
FUDR	Fluorodeoxyuridine
HAI	Hepatic artery infusion
HNPCC	Hereditary non-polyposis colorectal cancer
HPI	Hepatic portal infusion
IOUS	Intraoperative ultrasound

IP3	Inositol Triphosphate
MAPK	Mitogen activated protein kinase
MRI	Magnetic resonance image
NO	Nitric oxide
PBS	Phosphate buffered saline
PHR	Percentage hepatic replacement
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
PT	Pertussis toxin
RFA	Radiofrequency ablation
TGF-β	Tumour growth factor β
TNF-α	Tumour necrosis factor α
UICC	International Union Against Cancer
US	Ultrasound
VEGF	Vascular endothelial growth factor

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CHAPTER I

Review of Literature

Colorectal cancer is one of the commonest cancers in the West. Despite improvements in surgical and oncological treatment, survival at 5 years remains at 50% and one of the main factors for this poor outcome is the high rate of liver metastases. There is an increasing understanding of the underlying genetic and cellular biology of this disease. Endothelin-1 (ET-1) has a role to play in the development of colorectal cancer and its metastases. It may therefore be possible to use ET receptor antagonists in the treatment of colorectal cancer metastases. The introduction reviews current knowledge of ET-1 with special emphasis on its role in cancer generally and colorectal cancer specifically.

1.1 Endothelins

1.1.1 Introduction

A family of peptides found to cause coronary vasoconstriction in 1985 (Hickey *et al*, 1985) was subsequently isolated and identified in 1988 (Yanagisawa *et al*, 1988), and named endothelins (ET). There are three recognised members of this family - endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3) - which are 21 amino acid peptides and act as modulators of vasomotor tone, cell proliferation and hormone production in a variety of tissues. However, more recently Kishi (1998) described a 31 amino acid peptide with potent vasocontractile activity which was inhibited by both endothelin receptors A and B (ET_A and ET_B) specific antagonists. Collectively, the ET family ligands, precursors and associated enzymes and the ET receptors are called the "ET axis" (Nelson JB *et al*, 1999).

1.1.2 Structure

Preproendothelin-1 is 212 amino acids long and is processed to the 38 amino acid prohormone big endothelin-1, which is secreted and circulates in the plasma. The glycoprotein enzyme endothelin-converting enzyme (ECE)(Yanagisawa *et al*, 1988) cleaves big endothelin-1 between positions 21 (tryptophan) and 22 (valine) to generate ET-1.

1.1.3 Gene Regulation

The genes for ET-1, ET-2 and ET-3 have been localised to human chromosomes 6, 1 and 20 respectively (Bloch *et al*, 1989; 1991; Arinami *et al*, 1991). In general, the expression pattern of the ET genes is analogous to the tissue localisation pattern of ET receptors (Haynes *et al*, 1993) and suggests that ET is most likely a local mediator, acting as an autocrine or paracrine factor.

It is generally known that little, if any, ET-1 is stored in endothelial cells under physiological conditions and therefore must be made constitutively and then released from the cells. Endothelial cell expression of preproET-1 mRNA is known to be altered by a number of factors, including haemodynamic shear stress (Morita *et al*, 1993), hypoxia (Kourembanas *et al*, 1991), transforming growth factor-β (TGF-β)(Kurihara *et al*, 1989), tumour necrosis factor-α (TNF-α)(Marsden PA *et al*, 1992) and thrombin (Yanagisawa *et al*, 1988). Endothelin production is inhibited by agents that stimulate intracellular cGMP levels, including endothelium derived relaxing factor (EDRF), nitrovasodilators, prostaglandins and heparin (Saijonmaa *et al*, 1990; Boulanger *et al*, 1991; Kohno *et al*, 1992; 1993; Imai *et al*, 1993; Yokokawa *et al*, 1993)

1.1.4 Local Production

Endothelial cells appear to be the primary source of ET circulating in plasma. It is, however, possible that a variety of other cell types may release ET as happens in a wide range of cultured cells. The isoform of ET most prevalent in culture media and in body fluids or tissue is ET-1. ET-3 may be predominant in some tissues, particularly gut and brain. ET-2 has been difficult to detect by readily available methods due to cross-reactivity of most antibodies with ET-1 and therefore, little is known about it.

Localisation studies using molecular probes have shown that ET-1 is made where it is likely to have an effect, supporting the hypothesis that ET-1 is a paracrine factor. ET-1 produced by human umbilical vein endothelial cells and grown to confluent monolayers on acellular amniotic membranes has been shown to release predominantly into the basolateral compartment. ET-1 is thus released toward what would be the underlying vascular smooth muscle cells of the vessel intima (Wagner OF *et al*, 1992).

1.1.5 Clearance from Plasma

ET is thought to be removed from the circulation and extra-circulatory spaces by binding to its receptors (ET_B) followed by internalisation, uncoupling and enzymatic degradation. Because of this high capacity clearance mechanism, free and intact ET would be expected to be scarce in biological tissue or fluid, as measurements of ET levels by immunoassay have demonstrated.

1.1.6 Endothelin-1

Endothelin-1 (ET-1) is produced in both endothelial and vascular smooth muscle

cells. As much as 75% of ET-1 secretion from cultured endothelial cells is towards the vascular smooth muscle side of the cells, where it can bind to specific receptors to cause vasoconstriction. While it is unlikely that this pool of ET-1 contributes to the plasma concentration it remains, nevertheless, useful to measure plasma endothelin-1 levels as they have been found to correlate well with severity of disease, such as in congestive cardiac failure (Wei C-M *et al*, 1994). ET-1 probably has a role in the maintenance of basal vasomotor tone. The infusion of BQ-123, a selective antagonist of ET_A receptors, into the brachial artery of normal subjects caused progressive vasodilation and a 64% increase in forearm blood flow after one hour (Haynes WG *et al*, 1994). Although each of the endothelins is capable of producing vasoconstriction, ET-1 is the most potent vasoconstrictor known to man for it is 100 times more potent than noradrenaline on a molar basis. ET-1 also potentiates the vasoconstriction caused by noradrenaline (Tabuchi *et al*, 1989) and vice versa.

1.1.7 Endothelin-2 and Endothelin-3

Endothelin-2 (ET-2) is produced predominantly within the kidney and intestine but the cells of origin are unclear. It has no unique physiological functions as compared to ET-1.

Endothelin-3 (ET-3), like ET-1, circulates in the plasma. It has been found ubiquitously in the brain, the gastrointestinal tract, the kidneys and lungs.

1.2 Endothelin Receptors

The endothelins bind to two types of receptors (A & B) in mammalian tissue. The

receptors are members of the super family of receptors linked with guanine-nucleotide-binding (G) proteins and range from 45,000 to 50,000 daltons in size in various tissues. The amino acid structure of the two types of receptor is approximately 50% identical.

The human ET_A and ET_B receptor genes have been assigned to chromosome 4 (Hosoda *et al*, 1992) and chromosome 13 (Arai *et al*, 1993) respectively. A third receptor type, ET_C, which selectively responds to ET-3 has been identified and cloned in *Xenopus laevis* dermal melanophore (Karne *et al*, 1993). A mammalian equivalent has not been identified.

The greatest similarity between ET_A and ET_B receptors is in the trans-membrane regions and the intracellular loops. The greatest dissimilarities are found in the N-termini and extracellular loops 1 and 3. Alteration of the N-terminal extracellular region alters ET-1 binding to the human ET_A receptor (Hashido *et al*, 1992) and results in differential binding affinity for the latter (ET-1>ET-2>ET-3).

1.2.1 Localisation and Function

ET_A is relatively abundant in human lung, aorta and heart and much less abundant in liver, pancreas, kidney, brain, adrenal, gut and skeletal muscle (Hosoda *et al*, 1992; Hori *et al*, 1992; Elshourbagy *et al*, 1993). ET_B is relatively abundant in cerebral cortex, cerebellum, liver, kidney, lung and placenta and much less abundant in pancreas, gut, heart and skeletal muscle (Elshourbagy *et al*, 1993). In general, within the vascular wall, smooth muscle cells mainly express ET_A and endothelial cells mainly express ET_B receptors (Hosoda *et al*, 1991). However, this generalisation is somewhat controversial in the light of results from functional studies. Moreland (1994) concluded from their functional studies, using BQ-123 and

sarafotoxin S6c (a selective ET_B receptor agonist), that ET_A receptors predominate on the high pressure (arterial) side of the circulation and ET_B receptors predominate on the low pressure (venous) side of the circulation. Thus it seems clear that ET-1 can produce contraction in arteries (i.e., vascular smooth muscle cells) via either an ET_A or ET_B-mediated pathway.

1.2.2 Endothelin Receptor Nomenclature and Subclassification

Though only two mammalian endothelin receptors have been isolated, cloned and expressed to date, the existence of additional endothelin receptors and/or endothelin receptor subtypes have been postulated based on pharmacological studies that cannot be explained on the basis of only the ET_A and ET_B receptor.

ET_A receptor subtypes: Initial work relating to the classification of endothelin responses suggested that endothelin-induced vasoconstriction was mediated by the ET_A receptor situated on vascular smooth muscle cells. There are numerous examples demonstrating inhibition of ET-1 induced vasoconstriction and mitogenesis by the ET_A receptor antagonist BQ-123 (Ihara M *et al*, 1992a). Karaki and colleagues (1993) observed that some ET_A receptor mediated responses were insensitive to inhibition by BQ-123, and they therefore proposed the existence of ET_A subtypes. It was postulated that the ET_{A1} receptor represented a subtype which is sensitive to inhibition by BQ-123 while ET_{A2} is insensitive to this type of inhibition.

ET_B receptor subtypes: There are numerous examples of pharmacological data indicating the existence of anatomically separate and distinct ET_B receptor subtypes; an ET_B receptor on the endothelium that produces the release of nitric oxide to mediate vasodilation, and a subtype on vascular smooth muscle that directly mediates vasoconstriction (Warner TD *et al,* 1993a; b; Douglas SA *et al*, 1995). ET_{B1}

was the name given to those ET_B receptors upon which the compound IRL1038 acted as selective antagonist and the compound PD142893 acted as a non-selective antagonist (Warner TD *et al*, 1993a). The ET_{B2} agonists were not affected by either of those compounds (Sudjarwo *et al*, 1993). The ET_{B1} receptors are in the endothelium and bring about the release of relaxing factors, while the ET_{B2} receptors are found in vascular smooth muscle and when activated cause vasoconstriction.

Other endothelin receptors: The ET_C receptor displays more affinity with ET-3 than with ET-1. It appears to trigger nitrous oxide (NO) release (Karne *et al*, 1993). Until now no homologous receptor has been discovered for it in mammal cells.

1.2.3 Cellular Mechanisms

Attempts have been made to explain the mechanism of action of ET on vascular smooth muscle. The vasoconstrictor response is affected by two types of receptor, ET_A and ET_{B2}. This vasoconstriction is correlated with a maintained heightening of intracellular calcium (Ca^{2+}) produced via a two-phase mechanism. The binding of ET-1 to the receptor activates phospholipase C which hydrolyses phosphotidilinositol-4,5-biphosphate (PIP2) into two products, inositol triphosphate (IP3) and diacilglycerol (DAG). The initial increase in Ca^{2+} concentration is caused by rapid mobilisation of intracellular Ca^{2+} stores by IP3. This initial amount of Ca^{2+} entering would be able to depolarise the vascular smooth muscle sufficiently for this in turn to activate the L-type voltage-dependent Ca^{2+} channels. Contraction could also be partially affected by the action of another product of PIP2 hydrolysis, namely DAG.

In addition to the vasoactive effects, ET-1 promotes proliferation of vascular endothelial cells (via ET_B) and of vascular smooth muscle (via ET_A) and fibroblasts

(via both receptors) resulting in structural changes to the vessel wall. The mitogenic effect of ET-1 is attributed to stimulation of tyrosine residues as well as phosphorylation and activation of two other mitogen-activated protein kinases in the vascular smooth muscle (Golfman *et al*, 1993; Hirata *et al*, 1989; Bobik *et al*, 1990).

1.3 Endothelin Antagonists

Experimental and clinical evidence exists to support the importance of the development of drugs that block the production or actions of ET for use in cardiovascular medicine and oncology, particularly in conditions in which these peptides are clearly implicated.

The first ET receptor antagonists were of a peptide nature. In the development of these compounds, it was discovered that ET_A receptors recognise the tertiary structure of the N-terminal and C-terminal portion of the ET molecules. However, ET_B receptors only recognise the structure of the C-terminal portion. In 1991 based on these conclusions the first described ET antagonist, IRL1038, was developed; this was a selective in vivo inhibitor of ET_B receptors capable of antagonising ET-1 and ET-3-induced contractions in guinea pig ileum (Sakurai *et al*, 1993). The first attempt at obtaining a selective ET_A receptor antagonist was made by taking a fragment of ET peptide with a modification of the disulphide bridges and with the introduction of a diaminopropionic acid in the structure. The compound thereby obtained inhibited the ET-1-induced contraction, but not that induced by ET-3 in the pulmonary arteries (Spinella *et al*, 1991). Based on these studies the compound BQ-123 was introduced. The latter is now accepted to be the gold standard compound for ET receptor identification (Ihara *et al*, 1992). The potency of ET antagonists

varies according to what we consider to be their short or long term action. In this way it was discovered that BQ-123 totally blocks the production of IP3 provoked by ET-1 in vascular smooth muscle cells, but not the mitogenic effect on them.

Competitiveness studies show that this loss of long term activity is not related to the degradation of the compound, but rather with dissociation processes. Another ET_A receptor antagonist of a peptide nature which has been developed is the compound BQ-610. This compound antagonises cerebral vasoconstriction produced by subarachnoid haemorrhage, which is thought to be mediated by endogenous ET-1 acting through ET_A receptors (Zuccarello *et al*, 1994).

Non-peptide ET antagonists have also been developed. The first was the compound 50-235 that was a selective ET_A receptor agonist with no effect on ET_B (Mihara *et al*, 1993). Some of the later compounds developed did not have heightened selectivity for one receptor or the other and were therefore considered to be ET_A/ET_B receptor antagonists. The best known non-peptide antagonist is the compound Ro-47-0203, also known as Bosentan. Bosentan shows 20 to 50-fold selectivity for the ET_A receptor in studies of the inhibition of [^{125}I]ET-1 binding to recombinant human receptors. In functional studies, however, it was somewhat less selective (Clozel *et al*, 1994). Other studies have shown that the systemic administration of Bosentan to patients with severe chronic heart failure produces systemic, pulmonary and peripheral venous vasodilation and improved cardiac performance, without causing reflex tachycardia (Kiowski *et al*, 1995). On the other hand it should be pointed out that the compound BQ-788 is a non-peptide antagonist selective for ET_B receptors. This compound has been widely used in animal experiments for the characterisation of receptors which mediate the effects of ET. It can potentiate the arterial blood pressure increases induced by ET-1 and this is because it blocks the ET_B receptors

in the endothelium and vascular smooth muscle; as a result, their initial vasodilator effect is sometimes lost (Ishikawa *et al*, 1994).

A novel class of orally active non-peptide ET_A antagonists has been discovered by high throughput screening. The compounds are ET_A selective, are orally available and show a long duration of action (Riechers *et al*, 1996). One of these is the compound A-127722, the most potent ET receptor antagonist disclosed to date, which has a more than 1000-fold greater affinity for binding to human ET_A receptors than to human ET_B receptors. It has been shown to be orally bio-available in the rat, dog and monkey. In the conscious rat, A-127722 exhibits a dose-related inhibition of the blood pressure response to exogenous ET-1 (Opgenorth *et al*, 1996).

The use of ET antagonists in cancer has demonstrated some benefit and further potential. Ali (2000b) demonstrated the mitogenic effect of ET-1 on human colorectal cancer cell lines in vitro. This action was inhibited by the application of the ET_A antagonist BQ-123. Moraitis (1997) and a group led by Bagnato (1997) also demonstrated similar results whilst working on ovarian cancer cell lines in vitro. Similar results in prostate cancer cell lines underpin ongoing clinical trials using ET_A antagonists in prostate cancer (Zonnenberg *et al*, 2003; Carducci *et al*, 2003). The proliferation of three human pancreatic carcinoma cell lines expressing ET_A receptors was significantly reduced using a selective ET_A antagonist (Bhargava S *et al*, 2005). *In vivo*, the selective ET_A inhibition resulted in reduced angiogenesis as measured by lower microvessel densities (Bhargava *et al*, 2005). The biological mechanisms of ET-1 action in cancer are described further on in this thesis.

1.4 Endothelin-1 and Cardiovascular Disease

The endothelins are involved in the patho-physiological mechanism of a number of vascular conditions. Evidence now suggests a role for ET in sustained vasoconstriction as seen in hypertension and heart failure, local ischaemia as in myocardial infarction and acute renal failure and vasospasm as in Raynaud's disease and subarachnoid haemorrhage.

Possibly all isoforms of ET may be involved in the pathogenesis of hypertension through several effects. In addition to increasing central (Nishimura *et al*, 1991) and peripheral (Wong-Dusting *et al*, 1990) sympathetic activity, it stimulates the generation of renin, angiotensin II, aldosterone (Rakugi *et al*, 1990), as well as the generation of adrenaline (Boarder *et al*, 1989). Besides its vasoconstrictor and pressor effect, ET-1 has positive inotropic properties (Ishikawa *et al*, 1988; McClellan G *et al*, 1996) and an antinatriuretic action (Rabelink *et al*, 1994). In moderate to severe human essential hypertension the expression of ET-1 gene is increased though plasma levels of ET-1 are only slightly elevated in experimental and human hypertension. This may be due to the fact that ET synthesised in the endothelium is mostly released towards the underlying smooth muscle cells.

The plasma concentration of Big ET-1 correlates well with the severity of heart failure and is the most powerful predictor of outcome (Masson S *et al*, 2006).

Ischaemia can induce synthesis of endothelins. In humans, plasma endothelial levels are increased in acute myocardial infarction and unstable angina and the higher the plasma endothelin level in myocardial infarction and unstable angina the worse the prognosis (Miyauchi *et al*, 1989). Plasma ET levels on the third day after myocardial infarction are significantly related to mortality and plasma ET levels at 9

weeks after hospitalisation with unstable angina are significantly related to the incidence of further cardiovascular events (Omland T *et al*, 1994).

ET has been implicated in the acute renal failure and hypertension associated with nephrotoxic agents such as cyclosporin (Kon *et al*, 1990). The renal vasculature is very sensitive to ET and administration of ET antagonists 24 hrs after the ischaemic event still provided marked protection against severe acute renal failure (Gellai *et al*, 1995). ET-1 anatgonists also play a role in the treatment of patiernts with pulmonary hypertension (Barst RJ *et al*, 2006).

ET is a potent vasoconstrictor of isolated cerebral arteries and its effects are potentiated after subarachnoid haemorrhage (Alafaci *et al*, 1990).

1.5 Endothelin-1 and Cancer

ET-1 has been found to act as a mitogen for human epithelial carcinoma cell lines in vitro (Shichiri *et al*, 1991). The known ET-1 positive mitogenic effects affect fibroblasts and cancer cells (Kusuhara *et al*, 1989; 1990). Elevated levels of ET-1 have been found in specimens of cancer tissue including prostate (Nelson J *et al*, 1995), colon (Asham *et al*, 1997), breast (Patel *et al*, 1995) and thyroid (Donckier J *et al*, 2004). Patients with primary or metastatic colorectal and primary liver cancers are also known to have elevated plasma ET-1 levels (Shankar *et al*, 1996; Nakamuta *et al*, 1993). ET receptors are upregulated in specimens of prostate (Nelson J *et al*, 1995), lung (Zhao *et al*, 1995), breast (Wulfing P *et al*, 2004) and thyroid papillary (Donckier J *et al*, 2004) cancers. In *in vitro* systems, ovarian cancer cell lines produce ET-1 and possess ET_A receptors (Bagnato *et al*, 1997). Bagnato, Moraitis and others, working on ovarian cancer cell lines, also found that addition of

exogenous ET resulted in specific dose-dependent increases in cell number for ET-1 and ET-2 but not for ET-3. In the presence and absence of the exogenous peptides, the addition of BQ-123 reduced the cell number. This result is consistent with ET-1 acting via ET_A receptors as a positive mitogen (Moraitis *et al*, 1997; Bagnato *et al*, 1997) and is discussed further in section 1.5.2.

Tumourigenesis is dependent on the parallel development of a vascular supply (Folkman J, 1972). The vessels originate from the host circulation and their development correlates positively with the metastatic potential of the tumour (Folkman J, 1971; 1972). Recent studies have attempted to manipulate the vessel growth in several cancers with a view to inhibiting growth of the primary and subsequent metastases. ET-1, a recognised vasoconstrictor peptide, has been implicated as an angiogenic agent (Dawas *et al*, 1999, Knowles *et al*, 2005). With the recent development of ET-1 antagonists, the manipulation of angiogenesis within tumours may be possible.

1.5.1 The Role of Angiogenesis in Cancer Development

Most solid human tumours will exist in situ for a substantial length of time without the need for vascularisation (Folkman J in The Molecular Basis of Cancer, 1995). Vascularisation is necessary for progression of the neoplasm beyond 2-3mm^3 and its ability to metastasise (Folkman J, 1971; Weidner N, 1995). A "switch", occurring in some of the tumour cells, to an angiogenic phenotype initiates vascularisation (Folkman J in The Molecular Basis of Cancer, 1995). This "switch" involves a change in the steady state equilibrium between positive and negative regulators of microvessel growth (Dameron *et al*, 1994).

The process of tumour angiogenesis starts with vessels and tumour growing towards each other (Harris A, 1997). Driven by angiogenic factors (bFGF, VEGF, tumour necrosis factor-β and PDGF) and dampened by the down-regulation of certain angiogenic inhibitors (angiostatin, endostatin, platelet factor 4 and interleukin 1), these "driven" endothelial cells divide much more rapidly than normal endothelial cells. The endothelial basement membrane is degraded initiating new vessel sprouting. The new tumour vasculature is abnormal in that it is leaky, thus allowing passage of fibrinogen and plasminogen. Effectively, the normal process of angiogenesis is incomplete since maturation is halted. The regulatory imbalances may be the key to this derangement of the angiogenic process in tumours. The maturation would thus be inhibited at a primitive stage characterised by leaky vessel walls. Tsujii (1998) (working on *in vitro* model systems involving co-culture of endothelial cells with colon carcinoma cells) demonstrated that cyclo-oxygenase (a key enzyme required for the conversion of arachidonic acid to prostaglandins) plays an important role in the promotion of tumour related angiogenesis. The latter was inhibited by the application of non-steroidal anti-inflammatory compounds.
Neo-vascularisation will usually herald the onset of symptoms and tumours may then become clinically detectable yet it is at this stage that tumours become less accessible to chemotherapy (Folkman J, 1995). The tumours possess an increased interstitial pressure from the leaky tumour vessels and the absence of intra-tumour lymphatic drainage. Thus vascular compression and eventually central necrosis ensue. Chemotherapeutic agents are unable to penetrate the tumour, partly for this reason.
Growth and metastases of tumours are known to be dependent on neo-vascularisation for several reasons. A high degree of tumour vascularisation with

newly formed leaky vessels increases the chance for tumour cells to enter the circulatory system and metastasise (Folkman J, 1995). At the cellular level, neo-vascularisation augments tumour growth through a "perfusion" effect which allows metabolic exchange and a "paracrine" effect which supplies growth factors (Folkman J, 1971; Shalaby F *et al*, 1995). Perfusion is more efficient than diffusion in allowing metabolite and catabolite exchange in crowded tissue.

The angiogenic / tumourigenic effects of ET-1 could be mediated through the known mechanism of VEGF stimulation and enhanced endothelial mitogenesis in the existing tumour or vasculature. As mentioned earlier, selective ET_A receptor inhibition in vivo resulted in reduced angiogenesis as measured by lower microvessel densities (Bhargava S *et al*, 2005). Wulfing (2004) observed a significant positive correlation between microvessel density and ET expression. Moreover, expression of VEGF was found more often in tumours with over-expression of ET and its receptors. In ovarian cancer, ET-1 stimulated VEGF production through the ET_A receptor and selective inhibition of this receptor significantly impaired the angiogenic response (Salani D *et al*, 2000)

Inhibition of VEGF has shown potent anti-tumour effects in in vivo animal models (Millauer *et al*, 1996). Thus ET-1 antagonists would play their anti tumour role as both anti-angiogenic and anti-mitogenic agents.

A number of anti-angiogenic therapies are proving effective in combating solid tumours. The focus of the therapies has comprised inhibiting positive angiogenesis regulators, enhancing negative ones or directly targeting tumour endothelial cells. The latter technique has proven effectiveness as demonstrated by Huang *et al* (1997) who demonstrated complete tumour regression of neuroblastoma xenografts in athymic mice. Similarly, Pipili-Synetos *et al*(1995), working on the in vivo model of

angiogenesis of the chick chorioallantoic membrane (CAM) and the model of growth and metastasis in the Lewis Lung carcinoma in mice, obtained results suggesting that angiogenesis inhibitors (isosorbide 5-mononitrate and isosorbide dinitrate) inhibited tumour growth and metastasis. Furthermore, in rodents, anti-angiogenic therapy increases the delivery of chemotherapy to a tumour (Teicher *et al*, 1994). This is explained by the suggestion that anti-angiogenic factors unpack the mass of tumour cells (Folkman, 1995), thus lowering the tumour's interstitial pressure. Recently, anti-VEGF agents have, indeed, improved prognosis in patients with metastatic colorectal cancer, when added to standard chemotherapy (Kabbinavar FF *et al*, 2006).

1.5.2 Endothelin-1 and its Role in Tumourogenesis

Raised levels of ET-1 in specimens of various cancers are well established. Patients with primary or metastatic colorectal and primary liver cancers are also known to have elevated plasma ET-1 levels (Shankar *et al*, 1996; Nakamuta *et al*, 1993). ET receptor density is also altered with ET_A upregulated, as detected by autoradiography, in specimens of prostate, lung and colorectal cancers while ET_B is downregulated (Ali *et al*, 2000a, Nelson J *et al*, 1995, Zhao *et al*, 1995).

There are several reports indicating correlation between ET-1 expression and prognosis in cancer. In breast cancer the expression of ET-1 and both its receptors correlated with acquisition of malignant potential (Wulfing P *et al*, 2003). Univariate analysis has shown that ET-1 expression, in addition to sex, nodal metastatic involvement and stage, were significant predictors of poor survival and disease free survival outcome figures in non-small cell lung carcinoma (Boldrini *et al*, 2005). In colorectal cancer, a Cox regression model identified age, advanced stage and

plasma big ET-1 levels as independent variables of significant prognostic value for overall survival (Arun *et al*, 2004).

Immunohistochemistry has shown that ET-1 is over-expressed in specimens of colorectal (Asham *et al*, 1997) hepatocellular (Kar *et al*, 1995) and prostate cancer (Nelson *et al*, 1996). A study conducted in our centre has also shown that colorectal cancer patients, with or without liver metastases, have elevated plasma levels of ET-1 (Shankar A *et al*, 1998). This is in agreement with studies on patients with hepatocellular cancer (Nakamuta *et al*, 1993). Also of interest is the finding that ET_A receptors are upregulated in ovarian (Bagnato *et al*, 1999), colorectal (Ali H *et al*, 2000a), lung (Zhao *et al*, 1995) and meningioma / glioma (Harland *et al*, 1998) suggesting that autocrine or paracrine ET-1 loops contribute to cancer growth and progression (Shichiri *et al*, 1991; Moraitis *et al*, 1997). The frequent concomitant downregulation of ET_B receptors in the same cancer types (Nelson J *et al*, 1996), suggests that ET_B is not used for any cancer associated signals or even its apparent loss may remove some as yet unidentified inhibitory loop.

In vitro, several cancer cell lines have demonstrated proliferation in response to exogenous ET-1. The cell lines include colonic, ovarian, and meningioma (Kitagawa *et al*, 1994). This effect was reversed on further addition of an ET_A receptor antagonist as is also the case in pancreatic cancer cell lines (Bhargava *et al*, 2005). Ovarian cancer cell lines produce ET-1 and possess ET_A receptors (Bagnato *et al*, 1997). Moraitis (1997) working on two ovarian cancer cell lines, further found that addition of exogenous ET resulted in specific dose-dependent increases in cell number for ET-1 and ET-2 but not for ET-3. In the presence and absence of the exogenous peptides, the addition of BQ-123 reduced the cell number. This result is consistent with ET-1 acting via ET_A receptors as a positive mitogen. Downstream

events which propagate the mitogenic effect on ovarian cancer cells include EGFR transactivation and MAPK (Bagnato *et al*, 1999). Overall the known ET-1 positive mitogenic effects are not restricted to endothelial cells but also affect fibroblasts and cancer cells (Kusuhara *et al*, 1989; 1990).

Specifically in CRC, my field of research, the application of an ET_A antagonist reduced cell growth in at least one human colorectal cell line (Ali *et al*, 2000 b). A pilot study using a syngeneic rat model of liver metastases demonstrated that the intraportal administration of the ET_A specific antagonist, BQ-123, shortly after tumour cells were inoculated via the same route, resulted in significant reduction in tumour load (Asham *et al*, 1997). These results may be due to the inhibition of the mitogenic effect of ET-1 but there is strong evidence for the anti-angiogenic effect complimenting the former if not instigating it.

1.6 Colorectal Cancer

Colorectal cancer is the second commonest cause of cancer death comprising 10% of all cancer deaths in the UK. In England and Wales there are 17,000 deaths each year attributed to colorectal cancer (mortality stats: reviews of the register general on deaths by cause, sex and age in England & Wales1993 (revised 1994); Vukasin AP *et al*, 1990; Cancer Research UK 2005 http://info.cancerresearchuk.org/cancerstats /types/bowel/incidence). Of the annual incidence of 30,000 new cases per year 10-12,000 are rectal cancers (Cancer Research Campaign. Factsheet 18.1 1993, 2005 Cancer Research UK http://info.cancerresearchuk.org/cancerstats /types/bowel/incidence). This distinction is important for deciding on neoadjuvant and adjuvant therapy and for the sake of comparisons between outcomes. Within the colon about 50% of cancers arise in the left side and 25% in the right (Cancer

Research UK 2005 http://info.cancerresearchuk.org/cancerstats /types/bowel).

The aetiology of colorectal cancer is multifactorial. It is now accepted that there is an inherited and sporadic from. Approximately 5% of colorectal cancer cases diagnosed are attributable to an underlying hereditary colorectal cancer syndrome (Kwak EL *et al*, 2007). These include hereditary non-polyposis colorectal cancer (HNPCC) which is inherited in an autosomal dominant fashion. It arises due to mutations in mismatch repair genes, whose role is to repair mistakes in base pair matching during DNA replication. The clinical diagnosis of HNPCC families is based on the Amsterdam criteria I and II of the International Collaborative Group on HNPCC (ICG-HNPCC) (Vasen HFA *et al*, 1999). Familial adenomatous polyposis (FAP) is less common than HNPCC but is associated with a 100% risk of developing colorectal cancer. Again, it is inherited in an autosomal dominant fashion and is due to a mutation in the tumour suppressor adenomatous polyposis coli (APC) gene on chromosome 5q (Wu JS *et al*, 1998; Potter JD 1999). Other less common inherited colorectal cancers occur in patients with the autosomal dominant Peutz-Jeghers syndrome and juvenile poylposis.

Genetic aspects to the development of sporadic colorectal cancer tend to be similar to those found in FAP cancers and comprise mutations of the APC, K-ras and p53 genes. The latter mutations are found in more than 60% of all adenomas and carcinomas (Powell SM *et al,* 1992; Vogelstein B *et al*, 1988; Scott N *et al*, 1993; Kikuchi-Yanoshita *et al*, 1992). These mutations drive the adenoma carcinoma sequence. The remaining sporadic cancers are genetically closer to HNPCCs. There has been evidence that dietary factors such as lack of fibre and vegetables, and excess of animal fats are associated with an increased risk of colorectal cancer for some time now (Burkitt, 1971; Modan *et al*, 1975; Bjelke, 1973). There has also

been recent, reserved, enthusiasm about the inhibitory effect of COX-2 inhibitors on colonic adenoma and adenomatous polyp progression (Bertagnolli MM *et al*, 2006; Arber N *et al*, 2006).

Recent advances in colorectal cancer have focused on combinations of monoclonal therapies and chemotherapy which have resulted in better clinical outcomes than with either modality alone (Wadler S, 2007). These include the incorporation of cetuximab (blocks EGF binding to the EGFR) and the monoclonal antibody bevacizumab (anti VEGF-A) into the various chemotherapy regimes. These combinations show increased response rates and overall survival (Iqbal S *et al*, 2004; Giantonio *et al*, 2006; Kabbinavar FF *et al*, 2006).

1.7 Liver Metastases

1.7.1 Summary

Tumour metastasis is the leading cause of death in cancer patients. The distribution pattern of metastases can be predicted in part by the pattern of regional venous drainage. Most metastases develop in the first capillary bed encountered after discharge from the primary tumour. However, tumours also metastasise to distant locations that are not predictable based on blood flow patterns. The high proportion of bone metastases in breast, prostate and lung cancers are examples of selective homing of tumour cells to a specific organ.

The liver is often the first site of colorectal metastatic disease and may be the only site of spread in as many as 30-40% of patients with advanced disease (Weiss *et al*, 1986). The predilection for metastases to develop in the liver is partly due to the fact that the liver receives the portal drainage from the gastrointestinal tract via the

mesenteric veins. In several experimental tumour models of liver metastasis, metastatic cells first implant in the portal endothelium (Barberaguillem E *et al*, 1989). These then break off into a portal radical and re-seed close to the initial lesion . Such satellite formation close to a large liver metastasis is common with secondary tumours of the liver (August DA *et al*, 1985).

For unexplained reasons the right lobe of the liver is more likely to develop metastases than the left lobe (Holbrook RF *et al*, 1995). Macroscopically these metastases are paler in colour than surrounding normal tissue. They enlarge by concentric growth and may attain a large size occupying most of the liver.

Liver metastases may be found in as many as 35% of patients undergoing resection of a colorectal tumour and another 8-30% will subsequently prove to have liver involvement. Furthermore, 40-50% of patients die within 5 years of an apparently curative resection and the liver is the most frequent site of relapse (Scheele J *et al*, 1990; Gordon NLM *et al*, 1993; Stangl R *et al*, 1994; Jatzko *et al*, 1995). 25% of those who develop recurrences have them confined to the liver and therefore may be candidates for surgical resection (Adson MA, 1987). Unfortunately only 5% of all patients with colorectal cancer will ultimately benefit from such intervention (Wagner JS *et al*, 1984). This does, however, translate into 1000 patients treated each year in the UK.

Patients with liver metastases present in the late stages unless detected at the time of the primary cancer or are in a surveillance programme. The symptoms may be weight loss, upper abdominal discomfort and malaise. Evidence of liver failure such as ascites and jaundice are poor prognostic markers.

Inhibition of invasion constitutes a new class of targets for chemo-prevention. Intervention could commence between the period of tumour proliferation and the

onset of invasion, preventing the series of events leading to metastasis.

1.7.2 Detection and Imaging of Liver Metastases

The extent of liver involvement by metastases is an important survival determinant (Wagner JS *et al*, 1984) and, therefore, accurate staging of liver metastases provides a guide to the potential success of surgery. Several staging systems have been proposed (Gennari *et al*, 1986; Gayowski *et al*, 1994). The system modified from the International Union Against Cancer (UICC) and The American Joint Committee on Cancer (AJCC) recommendations for primary hepatobiliary tumours is in popular use. It incorporates tumour size, tumour distribution, number of metastases and extent of extra-hepatic disease (table 1.1).

Stage	5 year survival
I – unilobar solitary tumours of any size	61%
II – unilobar multiple tumours < 2cm without nodal disease	61%
III – unilobar disease with multiple lesions>2cm	28%
IVa – bilobar lesions	20%
IVb – bilobar lesions with nodal disease or extrahepatic disease	0%

Table 1.1 - The staging system modified from the International Union Against Cancer (UICC) and The American Joint Committee on Cancer (AJCC) recommendations for primary hepatobiliary tumours with predicted 5-year survival.

The identification of spread helps to target treatment for those with confined disease and provides valuable prognostic information to all patients with colorectal cancer. The most widely used imaging technique is a contrast CT scan of the abdomen with

detection rates for hepatic metastases of 68-91% (70% detection for lesions >1cm). An alternative to the transabdominal ultrasonography approach is intra-operative ultrasound examination (IOUS). IOUS may detect small lesions (<5mm) and other lesions indicating extra hepatic involvement, such as the porta hepatis lymph nodes, which may be difficult to visualise percutaneously (Ravikumar TS, 1996, Kruskal JB *et al*, 1996).

Early experience with laparoscopic ultrasonography suggests it can provide valuable staging information which may alter management (John TJ *et al*, 1994). The combination of a staging laparoscopy and laparoscopic ultrasound improve the selection of candidates for curative resection (Foley EF *et al*, 1998).

A recent meta-analysis of various imaging modalities for detection of colorectal metastases showed that F-18 fluorodeoxyglucose (FDG) PET had significantly higher sensitivity on a per patient basis but not on a per lesion basis compared with other modalities. Sensitivity estimates for MRI imaging with contrast agent were significantly superior to those for helical CT scan with 45 g of iodine or less (Bipat S *et al*, 2005). PET may still miss small hepatic lesions and its performance is affected by recent or current administration of chemotherapy. The list of imaging investigations used for diagnosing liver metastases is outlined in table 1.2. The colon should be fully examined to exclude recurrence or metachronous lesions. Also, the chest needs to be imaged for pulmonary deposits.

1.7.3 Current Management of Colorectal Liver Metastases - Surgery

Patients with untreated liver metastases generally have a poor prognosis, the median survival being less than 12 months (Bengtsson G *et al*, 1981; Lahr CJ *et al*, 1983) and none survive five years. However, survival from time of diagnosis is

Imaging Modality	References
CT	Ward 1989
Transabdominal ultrasound	Ward1989
Intra-operative ultrasound	Charnley 1991; Hagspiel 1995
Laparoscopic ultrasound	John 1994; Foley 1998
FDG-PET	Bipat 2005
PET / CT / Spiral CT	Votrubova J 2006
MRI	Bipat 2005
Hepatic flow scinitigraphy and duplex colour ultrasound (for calculation of hepatic perfusion index)	Leveson 1985; Leen 1991

Table 1.2 – Imaging investigations used in the diagnosis of colorectal liver metastases.

longer in patients with solitary lesions than in those with more widespread involvement (Rougier P *et al*, 1995). Several large series have reported five year survival ranging from 25% to 44% following resection of colorectal liver metastases (Cady B *et al*, 1991; Fong *et al*, 1999; Choti MA *et al*, 1999). A randomised study assessing outcome following resection compared with other treatment modalities is not likely to be undertaken.

Management of all patients with colorectal cancer should be the responsibility of colorectal cancer multidisciplinary teams. More recent data reports lower operative mortality after resection of colorectal liver metastases than that reported in previous decades (Scheele J *et al*, 1995; Stangl R *et al*, 1994; Cady B *et al*, 1991; Choti MA *et al*, 1999; Fong Y *et al*, 1997). No doubt, the reason for this is mutlifactorial but a volume effect on outcome has been shown by several recent studies (Dimick JB *et al*, 2003).

Resection of hepatic metastases from colorectal cancer has been shown to offer the best chance of long-term survival (Scheele J *et al*, 1995). Unfortunately the majority of patients with colorectal hepatic metastases are not suitable for resection and therefore specific criteria should be used to select those who may benefit from such intervention. There still remains some controversy amongst clinicians about which patients are most suitable for liver resection and which would be better treated by non-surgical means.

In 1963 Woodington and Waugh published their experience with resection of colorectal liver metastases at the Mayo Clinic and demonstrated that a 20% 5-year survival was possible in selected patients (Woodington GF *et al*, 1963); these results were confirmed by others who reported their experience of liver resection for metastases from a variety of gastrointestinal primary tumours (Flanagan L *et al*, 1967). Although only 7-10% of all patients with colorectal metastases are likely to benefit from hepatic resection, the frequency of this condition means that in the US approximately 5000 patients each year might be suitable for resection of liver metastases (Steele G *et al*, 1991); extrapolated to the UK this suggests that up to 1000 patients each year might be treated in this way with benefit. Attempts to increase resection rates with neo-adjuvant (pre-treatment) chemotherapy have shown promise with a 14% increase in resectability reported in one study (Adam R *et al*, 2001). Neo-adjuvant chemotherapy has been used to convert unresectable tumours to resectable ones using 5-FU, folinic acid and oxaliplatin. Concerns about the remaining liver reserve after resection have led to the use of preoperative portal vein embolisation to increase the volume of the residual liver (Farges O *et al*, 2003). Another approach has been to plan a two stage

hepatic resection in which the first stage is a non-curative resection or ablation and after subsequent hypertrophy the residual tumour is excised (Adam R *et al*, 2000; Neeleman N *et al*, 1996).

The two most important features of a tumour that influence patient survival in untreated liver metastases are extent of hepatic involvement at the time of diagnosis and the grade of the primary tumour. Multivariate analysis has not shown a consistent result regarding the relevance of the number of lesions on long term survival (Scheele J *et al*, 1995; Fong Y *et al*, 1997; Fong Y *et al*, 1999; Hughes KS *et al*, 1986; Schindl M *et al*, 2005).

It is, therefore, important to define the acceptable residual functioning volume. Acceptable residual liver volume is thought to be a third of the standard liver volume or the equivalent of two liver segments (Garden OJ *et al*, 2006).

Multivariate analysis of various prognostic factors provides a valuable guide to patient selection for hepatic resection and may prevent unnecessary surgery in patients unlikely to derive benefit from the latter (table 1.3).

Tumours that have the capacity to invade and metastasise to lymphatics are likely to be more aggressive than those without this potential. They may conceivably by-pass the liver, by spreading to pre-aortic lymph nodes, and beyond to the thoracic duct and systemic circulation. Although there may be a correlation between primary tumour stage and survival the relationship is not strong enough to preclude liver resection for patients with more advanced primary tumours. Despite this, there is again no consensus view on the correlation between stage of primary tumour and prognosis in published data (Scheele J *et al*, 1995; Nordlinger *et al*, 1992; Jatzko *et al*, 1995; Gayowski TJ *et al*, 1994).

Distribution of metastases	Gayowski 1994; Little 1987
Adequacy of resection margins	Registry of Hepatic Metastases; Hughes 1988; Little 1987
Formal resection or ennucleation	Hughes 1988; Scheele1995; Jatzko1995, Lise 1990
Age of patient	Fong 1995
Synchronous vs metachronous resection	Hughes1988; Nordlinger from his book; Lise 1990
Presence of extra-hepatic disease	Rosen 1992; Nordlinger from his book
Tumour stage and grade	Jatzko 1995; Gayowski 1994

Table 1.3 – factors influencing survival following resection of liver metastases

Survival following resection. The reported overall 5-year survival rates following hepatic resection of colorectal liver metastases range from 16-50% (Scheele J *et al*, 1995; Jatzko JR *et al*, 1995; Fong Y *et al*, 1999; Ruers & Bleichrodt, 2002)). Studies of the natural history of untreated liver metastases reveal that a minority of patients with limited disease may live for long periods without treatment. Benefit following resection of liver metastases may be gauged best by comparing survival after resection with survival in this group of patients (Wagner JS *et al*, 1984). Even though some of these patients have "favourable" tumour biology, possibly allowing prolonged survival without treatment, long-term survival beyond 5 years is rare without liver resection (Wagner JS *et al*, 1984). This contrasts to patients undergoing hepatic resection where a survival plateau is noted about 5 years after resection (Adson MA, 1987; Steele G *et al*, 1989); the surviving patients subsequently have a life expectancy similar to that of a matched non-cancer cohort (Adson MA, 1987). Repeat resection also seems to provide equal benefit to the first liver resection

(Antoniou A *et al*, 2007; Suzuki *et al*, 2001).

1.7.4 Other Therapies

Chemotherapy. Most patients with liver metastases are not suitable for resection and undergo palliative chemotherapy. Others receive adjuvant therapy and a smaller proportion receives neoadjuvant therapy aiming to down-stage unresectable tumours to resectable ones. Liver metastases receive up to 95% of their blood supply via the hepatic artery. Therefore hepatic arterial chemotherapy, through its locoregional effect, produces better response rate in patients with colorectal liver metastases. Some studies showed that this resulted in improved survival (Martin *et al*, 1990) and quality of life (Allen Mersh *et al*, 1994).

It is not possible to cure liver metastases with chemotherapy but the latter can prolong survival and should be considered in all patients not suitable for surgery (Scheithauer W *et al*, 1993; Simmonds PC, 2000; Jonker DJ *et al*, 2000). The chemotherapy may be administered systemically or via the hepatic artery or portal vein. The most commonly used systemic treatment, 5-fluorouracil (5-FU), inhibits thymidylate synthase and results in a tumour response in 5-18% of cases (Rougier P *et al*. In: Nordlinger B 1992). Newer agents such as irinotecan (topoisomerase I inhibitor) have shown improved 1-year survival compared with standard 5-FU regimen (Rougier P *et al*, 1998). The National Institute of Clinical Excellence (NICE) recommends the use of oxaliplatin-based regimens as first line therapy for all patients with non-resectable disease, and irinotecan based regimens for second line therapy after failure of first line treatment (Douillard JY *et al*, 2000; Giacchetti S *et al*, 2000).

Neoadjuvant chemotherapy has been used to convert unresectable tumours to

respectable ones using 5-FU, folinic acid and oxaliplatinum. A conversion rate from unresectable to resectable of 38% and a 3-year survival rate of 55% were reported (Bismuth H *et al*, 1996; Giacchetti S *et al*, 1999).

Adjuvant chemotherapy following liver resection uses 5-FU and folinic acid given systemically as a continuous infusion or a bolus. Two randomised studies show no difference in long term survival following hepatic artery infusion (HAI) (Rudroff C *et al*, 1999; Lorenz M *et al*, 1998). A much larger randomised trial comparing adjuvant HAI plus systemic chemotherapy versus adjuvant systemic chemotherapy in a total of 156 patients found that the former group had a significantly higher 2-year disease-free survival (Kemeny N *et al*, 1999).

Ablative therapies. The precise role of ablative therapy for hepatic metastases remains unclear. The CLOCC trial should help clarify this issue (http://www.eortc.be/protoc/Details.asp?Protocol=40004). For those who decline surgery, have associated co-morbidities that preclude resection or have treatable extrahepatic disease ablative therapy may be indicated. There may also be a role for ablative therapy in patients whose tumours have been downstaged but remain non-resectable (Oshowa A *et al,* 2003; Jansen MC *et al,* 2005; Elias D *et al*, 2003; Solbiati L *et al*, 2001). Resection combined with radiofrequency ablation (RFA) provides a surgical option for those with unresectable liver metastases and may prolong survival (Pawlik TM *et al*, 2003). RFA has also been shown to be effective on its own in the treatment of small liver metastases (Abitabile P *et al*, 2006) with 80% 2 year survival.

1.8 Aims

This project aimed to investigate:

1. The effect of ET-1 and its receptor antagonists on colonic cancer cell lines.
    a. a mechanism for this action was sought .
    b. ET receptor activation.
2. The ET receptor profile in human colorectal liver metastases tissue.
3. The *in vivo* effect of an oral ET_A receptor antagonist on CRC liver metastases in an animal model.

CHAPTER II

Mechanisms of Endothelin-1 Stimulated Proliferation in Cancer Cells

Aims

This chapter investigates the production of ET-1 and mechanisms of ET-1-stimulated proliferation in cancer cell lines. As described in the introduction ET-1 promotes a mitogenic stimulus in vitro in a number of cancer cell lines. In the majority of studies the mitogenic signal is transmitted through the ET_A receptor.

2.1 Introduction

Endothelin-1 (ET-1), a 21 amino acid vasoactive peptide (Yanagisawa *et al*, 1988), is a member of a family of three endothelins (Inoue *et al*, 1989). Its actions are mediated via two G protein-coupled receptor subtypes, ET_A and ET_B (Arai *et al*, 1990; Sakurai *et al*, 1990). ET-1 stimulates growth of smooth muscle (Komuro *et al*, 1988), fibroblasts (Yamashita et al, 1992) and several human cancer cell lines. The latter include ovarian (Bagnato A *et al*, 1999), prostatic (Nelson J *et al*, 1996), meningioma (Kitagawa *et al*, 1994) and CRC (Ali H *et al*, 2000 b) cells. ET_A receptor antagonists block the growth effect in human ovarian, prostatic and CRC cell lines. The present study investigates mechanisms of ET-1 induced growth and the roles of mitosis and apoptosis. Furthermore, the effect of G-protein inhibition on the ET-1 mitogenic effect was studied.

Two human CRC cell lines and one rat fibrosarcoma cell line were used. The latter was chosen as it was used previously in an animal model of colorectal liver metastases. In the latter model, the role of ET_A receptor antagonists *in vivo* was investigated and found to reduce tumour growth significantly (Asham E *et al*, 2001).

2.2 Materials and Methods

2.2.1 Cell Culture

Two human colorectal cancer cell lines; SW620 (derived from a metastatic colorectal adenocarcinoma, ECACC, Salisbury, Wiltshire, UK) and LIM1215 (derived from an HNPCC cancer donated by Professor M. O'Hare, Ludwig Institute of Cancer Research, London, UK) and a rat fibrosarcoma cell line MC28 (originally donated by Professor S. Eccles, Institute of Cancer Research, Sutton, Surrey, UK) were used. The cells were routinely cultured at 37 °C in Dulbecco's Modified Eagle Medium (DMEM) containing phenol red pH indicator. The medium was supplemented with 10% foetal calf serum (FCS), L-glutamine (2mM), penicillin (100IU/ml) and streptomycin (100µg/ml) (all reagents bought from Imperial Laboratories, Basingstoke, Hants, UK). The cells were incubated until they reached 80-90% confluence. At this stage the cells were routinely disaggregated with trypsin (1mg/ml in 0.02% EDTA (ethylenediaminetetraacetic acid) in PBS (phosphate buffered saline)), counted and either passaged or used for the experimental studies described below.

2.2.2 ET-1 Assays

On reaching confluence in 75cm^3 flasks, the cells were disaggregated and seeded at a 1:3 split in FCS-containing medium. After 24 hours the cells were incubated in serum free medium (containing glutamine and antibiotics as described above). Supernatants were collected at 24 and 48 hours, spun down to exclude dead cells and debris then stored at -80°C. At the time of supernatant collection the adherent cell numbers were counted using a haemocytometer. ET-1 levels in the media were assayed using an ET-1 kit for an ELISA (Nycomed Amersham, Little Chalfont,

Bucks., UK). The levels were expressed per ml of medium per 10^6 cells.

2.2.3 Growth Assays

Measurement of cell growth was based on changes in cell number. Responses to the addition of exogenous ET-1 (Sigma Laboratories, Poole, Dorset, UK) were investigated under serum-free (0% FCS) conditions. Cells were plated in 24 well plates at a concentration of 2.0 x 10^4 cells/ml/well in fully supplemented DMEM. After 24 hours the cells were washed (PBS), and incubated in 1ml/well of ET-1 (in concentrations ranging between 10^{-11} to 10^{-7} M) in serum-free (0% FCS) medium. After 48 or 72 hours the cells were washed and growth stopped by the addition of 10% formal saline at 4°C for at least 1 hour.

In the experiments where ET receptor antagonists were used, cells were seeded, as before, for 24 hours. Cells were washed in PBS and then exposed to ET-1 at its most effective dose (determined from the previous experiments for each cell line) as well as one of two ET receptor antagonists; A-127722 (Alexis Corporation, Bingham, Notts., UK), a selective ET_A receptor antagonist (at concentrations of 10^{-11} to 10^{-7} M) or BQ-788 (Alexis Corporation), a selective ET_B receptor antagonist (at a concentration of 10^{-8} M), for 48 or 72 hours. Cell growth was stopped by fixation with formal saline as above.

The effect of G-protein inhibition with pertussis toxin (PT) on ET-1 stimulated cell growth was assessed on LIM1215 and SW620 only. Cells were pre-treated for 6 hours with PT then exposed to increasing doses of PT (0.1-100ng/ml)±ET-1 (10^{-8}M) for 48 hours. Again cells were fixed in formal saline, as above.

Cell number was then determined by the methylene blue assay (Oliver MH *et al*,

1989) and read as absorbance at 650nm. Briefly, the dye chelates in nucleic acids, therefore the larger the amount of nucleic acids (i.e the larger the cell number), the higher the absorbance values.

2.2.4 Immunohistochemistry

DNA replication (as a measure of proliferation) in cells grown in the presence of ET-1 and/or ET_A receptor antagonist for 48 or 72 hours in 24 well plates was determined by immunohistochemistry. The protocol followed was described in the previous section, and concentrations used for ET-1 were10^{-8}M for MC28, 10^{-9}M for LIM1215 and 10^{-7}M for SW620 and the concentrations of A-127722 were 10^{-11}M for MC28, 10^{-9}M for LIM1215 and 10^{-7}M for SW620. The labelling reagent, 5-bromo-2'-deoxyuridine (BrDU), (Dako Ltd., High Wycombe. Bucks, UK) which binds to replicating DNA, was diluted 1:1000 in serum free tissue culture medium. Medium from the cultured cells was replaced with medium containing BrDU and cells were incubated at 37°C for 6 hours. The labelling medium was then removed and the cells washed in phosphate buffered saline (PBS). The cells were fixed in acid-alcohol (90% ethanol, 5% acetic acid, 5% water) at room temperature for at least 30 minutes. The wells were re-hydrated by washing in PBS and then incubated in reconstituted nuclease/anti-5-bromo 2'-deoxyuridine (Dako Ltd.) for 1 hour. After washing, the cells were incubated in peroxidase anti-mouse IgG2a for 30 minutes, washed again and incubated in 3,3'-diaminobenzidine tetrahydrochloride (DAB) for 5 to 10 minutes. The proportion of cells stained with DAB was counted manually (approximately 6 fields per slip), under a microscope, by two independent observers. The stained cells were those in which replicating intracellular DNA had bound BrDU. Our own pilot studies demonstrated that less than 4 hours incubation with BrDU

resulted in a very small proportion of stained cells, while incubations over 10-12 hours resulted in the majority of cells stained. Therefore, optimum exposure was determined at 6-8 hours.

2.2.5 Flow Cytometry

Apoptotic cells were recognised by their ability to bind Annexin V on cell-membrane-exposed phosphatidyl serine. These cells were then identified by flow cytometry. Cells grown in DMEM and exposed to ET-1 and/or specific endothelin receptor antagonists were harvested with trypsin and washed once in Annexin V binding buffer (BD PharMingen, San Diego, CA, USA). The cells were re-suspended in 1ml HEPES Buffer and 5.5µl of Annexin V-FITC (BD PharMingen) was added. After incubation in the dark at room temperature for 20 minutes, 100µl of a 50µg/ml solution of Propidium Iodide (Sigma) was added to gate out the dead cells.
The cells were analysed by flow cytometry using a FACSCalibur (Becton Dickinson, San Jose, CA, USA) measuring Annexin-FITC fluorescence in the FL1 channel (530/30 bandpass filter) and Propidium Iodide in FL2 (585/42 bandpass filter). 20,000 events were collected. Apoptotic cells were defined as being Annexin positive but Propidium Iodide negative, while late apoptotic/necrotic cells were positive for both Annexin and Propidium iodide.

2.2.6 Statistical analysis

Analysis of all cell culture experiments was carried out using a 1-way ANOVA. If statistically significant, post-hoc analysis was undertaken. A one-way Dunnetts test, set at $p<0.05$ was used for ET-1 dose response experiments, and Tukeys honestly significant difference (HSD) test, also set at $p<0.05$, was used for all other analyses.

Means of minimum 6 independent repeats for all experiments were analysed, unless stated otherwise. All graphical data are shown as means and standard deviations (SD). Immunocytochemical data are given as medians ± ranges; the Mann-Whitney test was used for analysis with significance set at 0.05.

2.3 Results

2.3.1 ET-1 Assay

The production of ET-1 by the rat fibrosarcoma cell line MC28 and the colorectal cancer cell lines SW620 and LIM1215 were measured in media conditioned for 24 and 48 hours. The normal range for ET-1 is 0.2 to 0.7 fmol/ml cells in human EDTA plasma and the following results were corrected for this. MC28 cells produced ET-1 to the value of 1.1 and 1.3 fmol/ml/10^6 cells at 24 and 48 hours respectively. LIM1215 values were 21.3 and 22.6 fmol/ml/10^6 cells respectively. SW620 ET-1 production measured 11.3 and 8.4 fmol/ml/10^6 cells respectively. The baseline reading for ET-1 amounts in medium only was 0.6 fmol/ml. Results were derived after pooling supernatant and cell numbers from 4 independent repeats. The assay was repeated once and a mean value derived.

2.3.2 Growth Assay

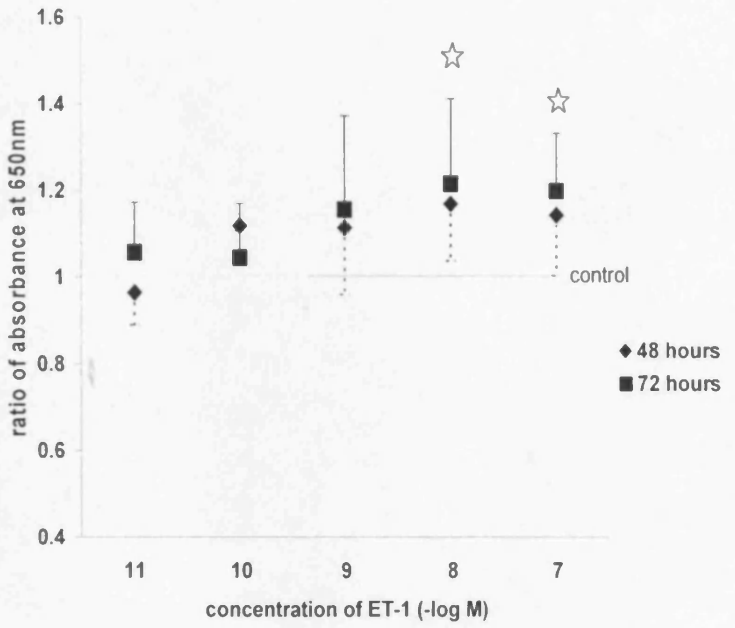
MC28 cells showed an increase in number of cells on addition of ET-1 (10^{-7} -10^{-10}M). The biggest effect was seen at an ET-1 concentration of 10^{-8}M with increases of 16.8% and 21.5% at 48 hours and 72 hours respectively (figure 2.1). The selective ET_A antagonist A127722 by itself did not influence cell growth at any concentration and neither did the selective ET_B antagonist BQ-788. The stimulatory effect of ET-1 (at the most effective growth-promoting concentration 10^{-8}M) was reversed by the

addition of the ET_A receptor antagonist, A-127722, at a concentration of 10^{-11}M (figure 2.4). No significant change was seen on addition of ET-1 and the ET_B receptor antagonist, BQ-788 (table 2.1).

LIM1215 cell growth showed similar trends to MC28 cells. The maximal effect of ET-1 was at 10^{-9}M (figure 2.2) showing an increase of 11.1% and 14.4% at 48 and 72 hours respectively. Neither antagonist by itself at any concentration had an effect on cell growth. Again the stimulatory effect of ET-1 (10^{-9}M) was reversed by A-127722 (figure 2.5). As previously, no effect was seen with BQ788. SW620 cell growth was augmented by ET-1 at concentrations of 10^{-7}-10^{-11}M. This effect was maximal at 10^{-7}M showing increases of 14.1% and 38% at 48 and 72 hours respectively (figure 2.3). A-127722 alone had a dose dependent inhibitory effect in its own right particularly at 10^{-7}M (figure 2.7), but BQ788 did not. The stimulatory effect of ET-1 (10^{-7}M) on SW620 cell growth was reversed by addition of A-127722 (figure 2.6). The maximal inhibition of growth was by 12.8% and 18.3% at 48 and 72 hours respectively, at an A-127722 concentration of 10^{-7} M. BQ788 was ineffective either way.

The mitogenic effect of ET-1 (10^{-8}M) at 48 hours was significantly reduced to near control levels on pre-incubation of cells for 6 hours with the selective G-protein antagonist PT. at doses of 1-100ng/ml, PT inhibited ET-1 stimulated growth to near control levels in both cell lines (SW620 and LIM1215). Inhibition was significant with doses of PT above 0.1ng/ml ($p<0.01$) (figure 2.8).

Figure 2.1 **MC28**



Figures 2.1-2.3: The effect of the addition of endothelin-1 (ET-1) on MC28 (figure 2.1), SW620 (figure 2.2) and LIM1215 (figure 2.3) cell growth at 48 and 72 hours of incubation. The cells were incubated in the presence of ET-1 (10^{-11} to 10^{-7}M; x axis). Cell growth was measured using the methylene blue assay and read as absorbance (equivalent to cell number) at 650nm. The absolute absorbance values were then converted to ratios (experimental / control; control=1). The results are displayed as mean ratio (S.D) (y axis) and for comparison a line through the control value is shown. Statistically significant growth is shown as ☆$p<0.05$ (1-way ANOVA, Dunnetts). The biggest effect on MC28 is seen at ET-1 of 10^{-8} M and this concentration was used for further experiments. For SW620 the biggest effect was seen at ET-1 concentrations of 10^{-7}M, and for LIM1215 it was 10^{-9}M. The data represents the mean (+SD) of 8 independent repeats.

Figure 2.2

SW620

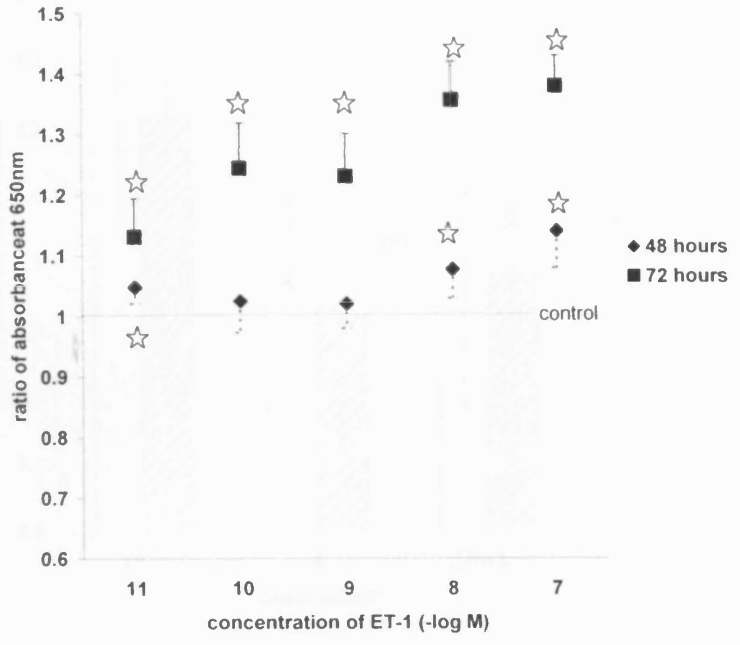


Figure 2.3

LIM1215

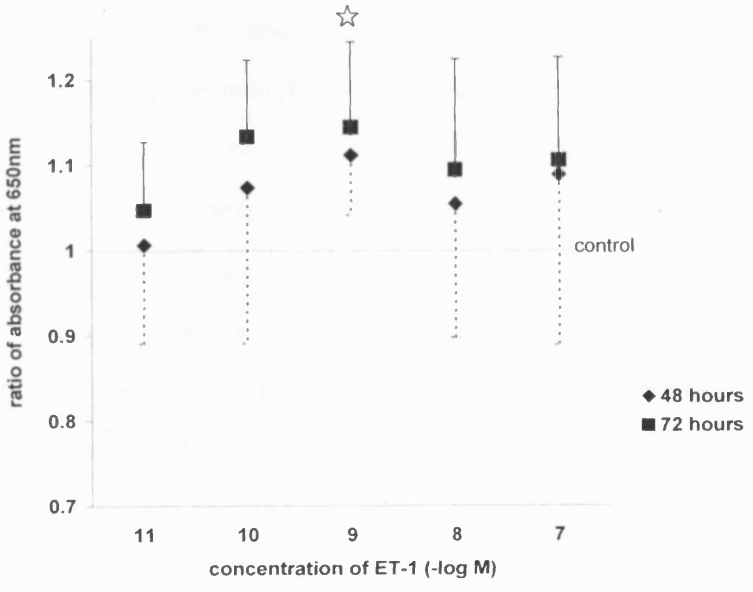
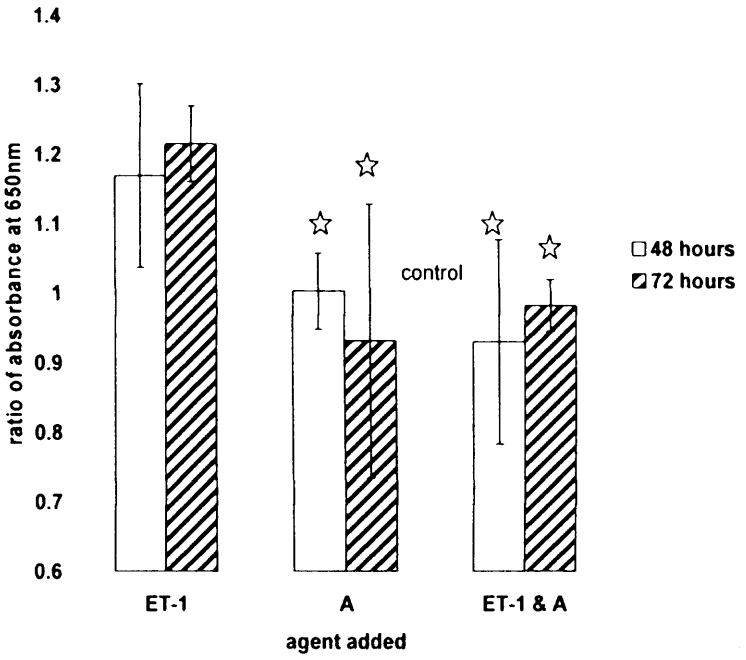


Figure 2.4 **MC28**



Figures 2.4-2.6: MC28 (figure 2.4), SW620 (figure 2.5) and LIM1215 (figure 2.6) cells were incubated in the presence of ET-1 (most effective concentration) ± ET_A (A-127722, A) or ET_B (not shown) receptor antagonists, or in the presence of ET_A antagonist (A) alone (x axis). Cell growth was measured using the methylene blue assay and read as absorbance (equivalent to cell number) at 650nm. The absolute absorbance values were then converted to ratios (experimental / control; control=1). The results are displayed as mean ratio (S.D) (y axis) and for comparison a line through the control value is shown. On addition of a combination of ET-1 and ET_A antagonist the increase in growth is reversed (☆ $p<0.05$ 1-way ANOVA, Tukeys HSD) in all three cell lines. For SW620 only (figure 2.5), the addition of the ETA antagonist by itself reduced growth significantly compared to control values (☆☆ $p<0.05$ Mann Whitney test).The concentrations of ET-1 used were 10^{-8}M, 10^{-7}M and 10^{-9}M for MC28, SW620 and LIM1215 respectively. The concentrations of ET_A antagonist used were 10^{-11}M for MC28 and LIM1215 and 10^{-7}M for SW620.

Figure 2.5 **SW620**

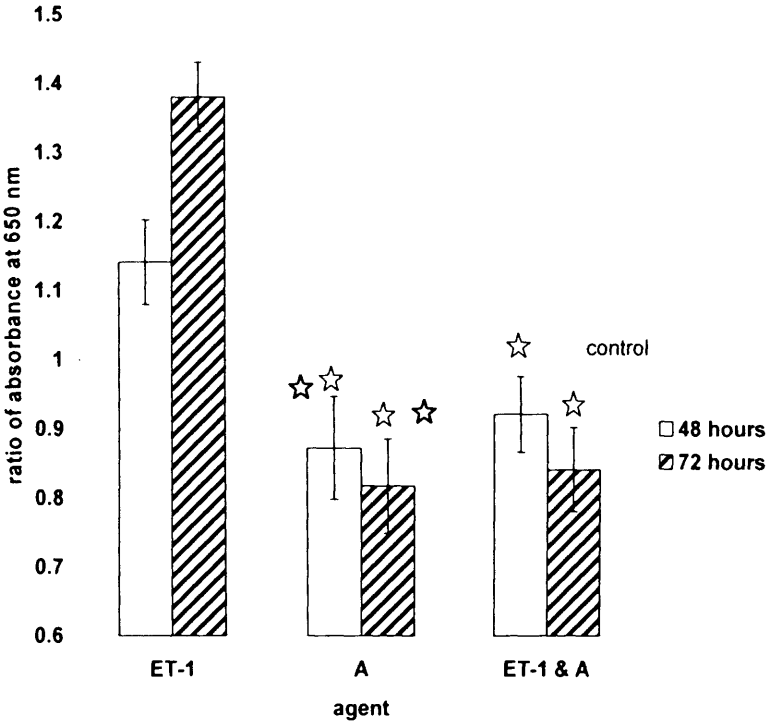


Figure 2.6 **LIM1215**

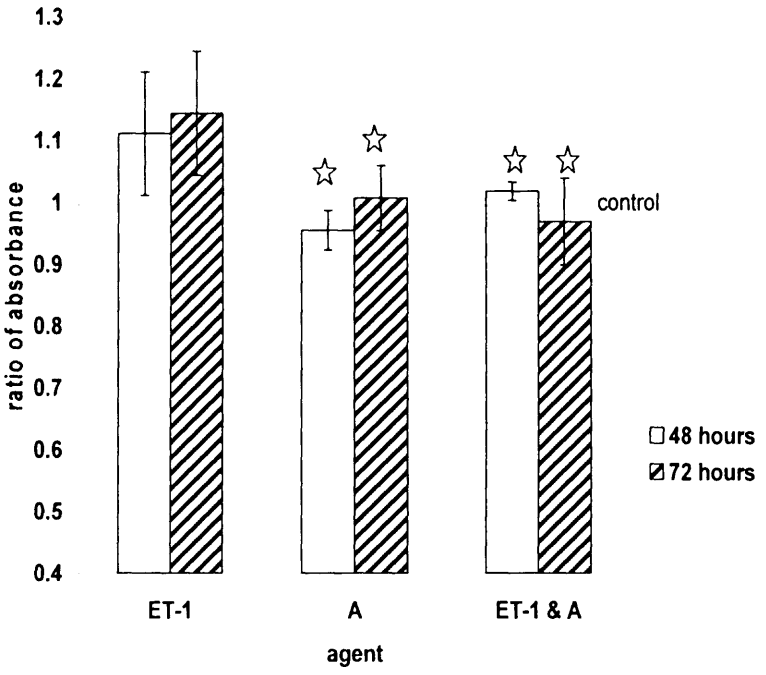


Figure 2.7 **SW620**

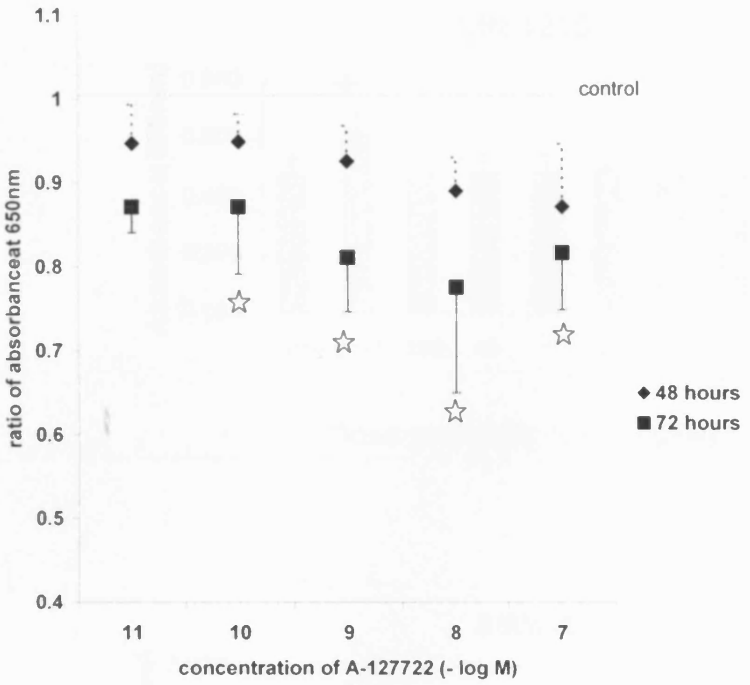


Figure 2.7: The effect of the addition of the ET_A selective antagonist A127722 on SW620 cell growth at 48 and 72 hours of incubation. The cells were incubated in the presence of A127722 (10^{-11} to 10^{-7}M; x axis). Cell growth was measured using the methylene blue assay and read as absorbance (equivalent to cell number) at 650nm. The absolute absorbance values were then converted to ratios (experimental / control; control=1). The results are displayed as mean ratio (S.D) (y axis) and for comparison a line through the control value is shown. Statistically significant growth is shown as ☆ $p<0.05$ (1-way ANOVA, Dunnetts). There was dose dependent inhibition, maximal at 10^{-7}M. The data represents the mean (+SD) of 8 independent experiments.

Figure 2.8

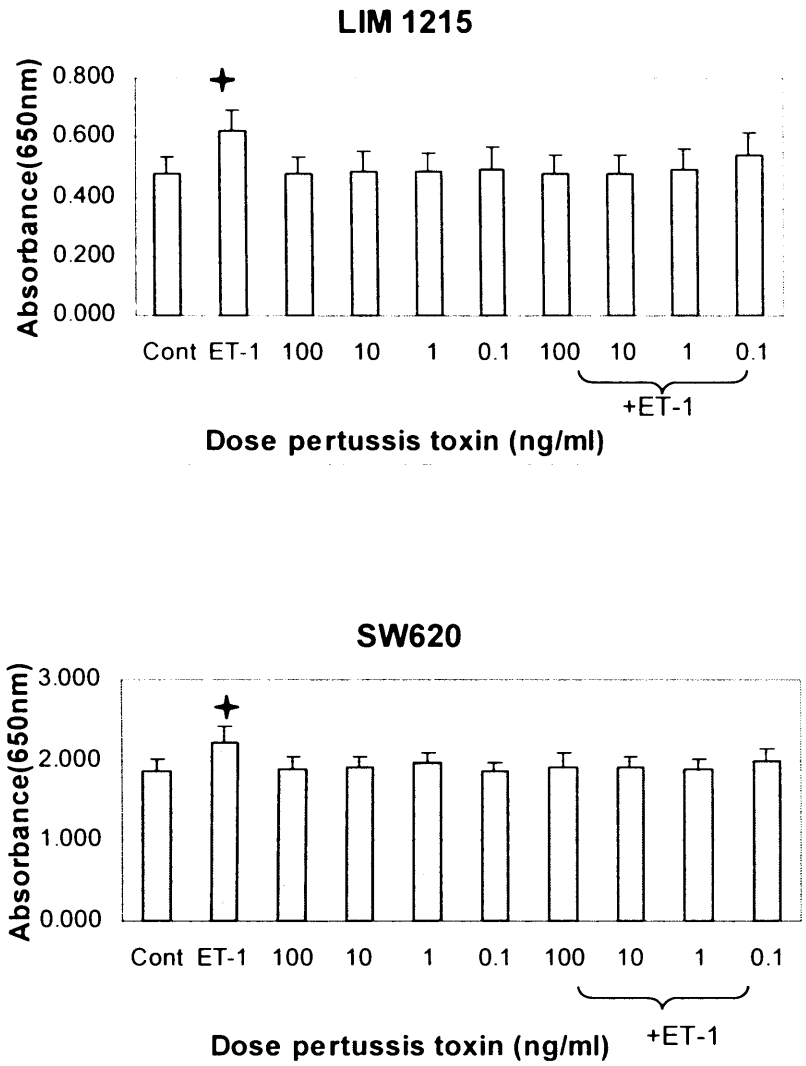


Figure 2.8: Effect of G-protein inhibition with pertussis toxin (PT) on ET-1 stimulated cell growth. Cells were pre-treated for 6hrs with PT, then with increasing doses of PT (0.1-100ng/ml) ± ET-1 (10^{-8}M) for 48hrs. Cell growth was measured using the methylene blue assay and read as absorbance (equivalent to cell number) at 650nm. Data represents the mean ± SD of six independent experiments. LIM1215: p=0.003, SW620: p=0.002 (Tukeys HSD). ✦, Significant at $p<0.05$ on post-hoc analysis (Tukeys HSD).

2.3.3 Immunohistochemistry

All cell lines showed significant ET-1-induced increases in DNA proliferation. These increases were reversed on further addition of the selective ET_A antagonist A-

127722.

The proportion of MC28 cells that stained for BrDU (displaying active DNA replication) under control conditions was 51.7% (S.D. +/-3.3). On addition of ET-1 the proportion became 64.3% (S.D. +/-1.3) (a 23% increase, $p<0.01$) and this returned to approximately baseline levels, 49.4% (S.D. +/-1.1), when the cells were incubated in both ET-1 and A-127722 (table 2.2).

LIM1215 cells followed the same trend. Under control conditions 42.1% (S.D. +/-2.1) of cells stained for BrDU, while 69.7% (S.D. +/-2.1) did so under the influence of ET-1 (67% increase, $p<0.01$). When ET-1 and A-127722 were added 38.3% (S.D. +/-3.6) of cells stained (table 2.2).

36.5% (S.D. +/-2.4) of SW620 cells stained for BrDU under control conditions. This percentage increased to 43.0% (S.D. +/-0.6) on addition of ET-1 (16% increase, $p=0.015$) and fell back to 37.4% (S.D. +/-2.3) on addition of both ET-1 and A-127722 (table 2.2).

2.3.4 Flow Cytometry

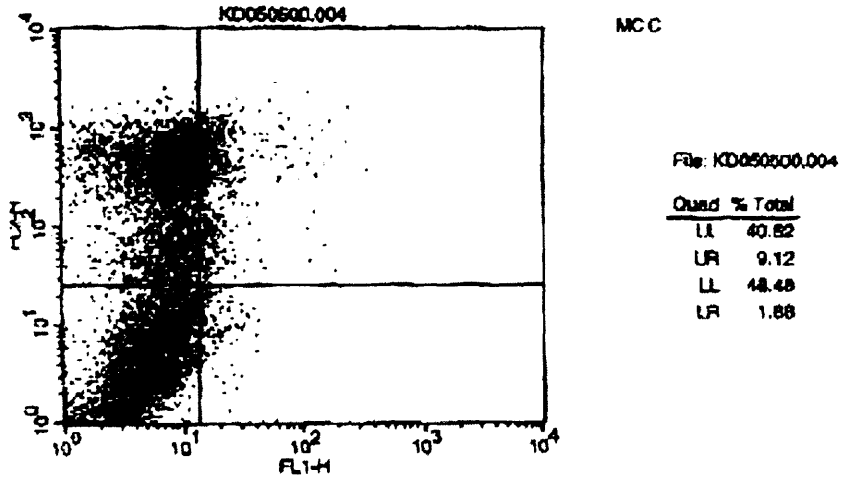
Viable cells do not bind Annexin V-BIOTIN or propidium iodide (lower left quadrant). Early apoptotic cells with exposed phosphatidyl serine but intact cell membranes bind Annexin V-BIOTIN but exclude propidium iodide. Fluorescence from this population is displayed in the lower right quadrant. Necrotic or apoptotic cells in terminal stages will be both Annexin V-BIOTIN and propidium iodide positive and are displayed in the upper right quadrant. A small percentage of normal cell death should be expected in routine cultures of untreated cells.

Figure 2.9 displays flow cytograms for MC28 cells. There were no significant changes in the number of cells undergoing apoptosis in the cells exposed to ET-1 (b) as compared to controls (a). Furthermore, the combination of ET-1 and A-127722

Figure 2.9. Flow cytograms for MC28 cells. Viable cells are shown in the lower left-hand quadrant of the dot plots (LL). Early apoptotic cell fluorescence is reported in the lower right-hand quadrant (LR). Necrotic or apoptotic cells in terminal stages will be reported in the upper right-hand quadrant (UR).

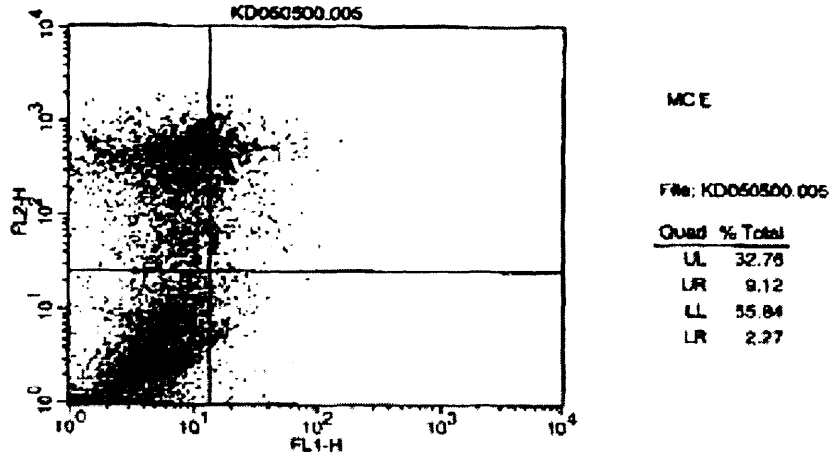
The cells were incubated in DMEM (a), in the presence of 10^{-8} M ET-1 (b) or in the presence of 10^{-8} M ET-1 and 10^{-11} M ET_A antagonist, A-127722 (c). The percentage cells in the LR

A

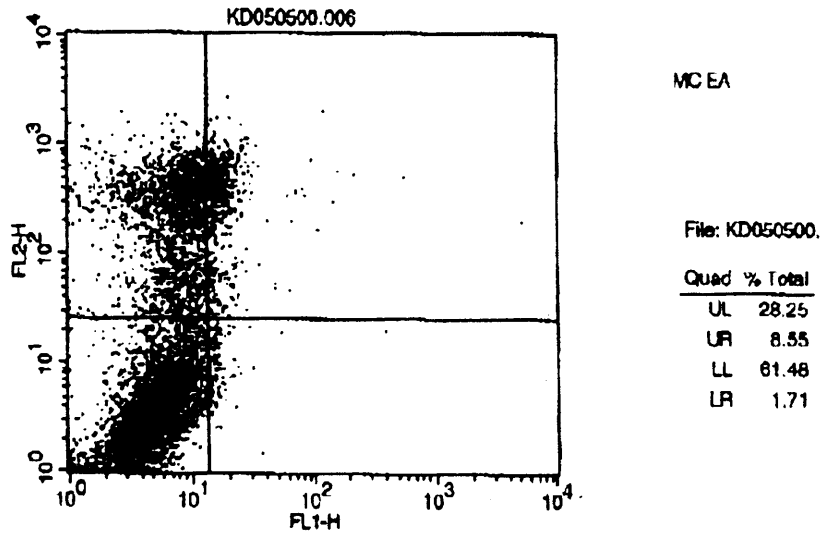


quadrant were 1.88, 2.27 and 1.71 respectively. This experiment was repeated 10 times for MC28 cells. Similar results were observed in the other cell lines.

B



C



(c) shows no change either. The results for all cell lines are displayed in table 2.3 and show similar results for LIM1215 and SW620.

2.4 Discussion

Endothelin-1 has mitogenic effects on several cancer cell lines including ovarian (Shichiri M *et al*, 1991; Bagnato A *et al*,1999), prostatic (Nelson *et al*, 1996), meningioma (Kitagawa et al, 1994), epithelial carcinoma cell lines derived from human cervix and larynx (Harland S *et al*, 1998) and colorectal (Ali H *et al*, 2000 b). This chapter describes a proliferative effect on colorectal cancer and rat fibrosarcoma cell growth.

The cancer cell lines investigated, all secreted varying concentrations of ET-1 into their medium. The highest concentration was produced by LIM1215 cells and the lowest by MC28 cells. In contrast, the ET-1 plasma level in patients with colorectal cancer has been measured at about 4 pg/ml (Shankar A *et al*, 1998). This represents a 10,000 fold increase compared to ET-1 from cancer cell lines.

However, ET-1 is thought to act in an autocrine or paracrine manner and, therefore, plasma levels do not necessarily give a true indication of action at the cellular level. The fact remains though, that plasma circulating ET-1 levels are in vast excess to those sufficient for cellular action.

In vitro, the biggest growth effect of ET-1 was seen on SW620 cells. Interestingly, only SW620 cells reacted to the addition of A-127722 alone. These results may be due to a relatively lower proportion of endogenous ET-1 binding to the receptors in SW620. Thus, the competitive exogenous ET-1 or A-127722 may have a more pronounced effect than on the other cell lines. Alternatively SW620 cells may have a larger density of ET_A receptors compared to the other two cell lines investigated or that there are different subtypes of the ET_A receptor with varying response intensities to the receptor antagonists.

The effect of ET-1 is mediated by the ET_A receptors. This is demonstrated by the reversal of the ET-1 growth effect with the addition of ET_A receptor antagonists. ET_B receptor antagonists have no effect. The addition of exogenous ET-1 had a much greater effect on cell growth than endogenous ET-1 since the use of the ET_A receptor antagonist, A-127722, had no inhibitory effect on endogenous ET-1 except when used on SW620 cells. These observations are supported by work on other cancer cell lines including prostate (Nelson *et al*, 1996) and ovarian (Kitagawa *et al*, 1994). However, there are reasonable suggestions that in both breast cancer (Rajeshkumar *et al*, 2005) and cutanous melanoma (Bagnato *et al*, 2004) the ET_B receptor plays a more integral role in mitogenesis than ET_A receptor.

Changes in cell numbers are a result of the interplay between apoptosis and mitosis. The increase in BrDU uptake suggests that ET-1 stimulates mitogenesis in the cancer cell lines. Radiolabelled thymidine incorporation studies performed by other

workers have also shown an increase in proliferation. Bagnato (1997) working on human ovarian carcinoma cell lines and Kitagawa (1994) working on cultured human meningioma cells both showed dose dependent increases in DNA synthesis in the presence of ET-1. This increase, in both cell lines, was blocked by BQ-123, an ET_A receptor antagonist. Measuring DNA replication using BrDU offers only a snapshot in time. However, preliminary measurements made after 8 hours of cell incubation with BrDU showed a similar result, while incubation for an hour was insufficient to show staining and 24 hour incubation resulted in the staining of all cells.

The flow cytometry data showed no changes in apoptosis after incubation with ET-1. However, ET-1 induced inhibition of apoptosis has been reported in endothelial cells via ET_B receptors (Shichiri M *et al*, 1998) and in prostate cancer cells via the ET_A receptor (Nelson J *et al*, 2005). Eberl and colleagues reported that the mixed endothelin receptor antagonist Bosentan promoted apoptosis in human (HT29 and SW480) and rat (PROb and REGb) colonic carcinoma cells *in vitro* (Eberl LP *et al*, 2000b). The latter study focused on changes detected 24 hours after treatment. In our experience at such a time interval there was no significant increase in net cell number growth. We, therefore, concentrated on findings at 48 hours and longer.

The G-protein inhibitor PT inhibited the mitogenic effect of ET-1 at doses 1-100ng/ml. This suggests that the mitogenic effect in colorectal cancer cells is mediated by PT sensitive G-protein sub-units: Go or Gi. Our findings contrast with results in ovarian cancer cell lines which demonstrated that ET-1 stimulated DNA replication was not inhibited by pre-incubation with PT (Bagnato A *et al*, 1997). The ET-1 mitogenic action in ovarian cancer cells, therefore, appears to be mediated via different G-protein subunits. In addition the mitogenic effect of ET-1 on vascular smooth muscle cells, mediated through the transactivation of the EGFr, requires a

pertussis toxin insensitive G-protein (del Bufalo D *et al*, 2002). However, stimulation of the ET_A receptor with ET-1 results in receptor coupling with the PT sensitive Go α and Gi_3 α-subunits, in addition to the PT insensitive G-proteins Gq/G_{11} (Iwasaki H *et al*, 1998).

ET-1 plays a critical role in tumourigenesis and Kim (2005) has shown that enhancement of the β-Catenin signaling pathway activates the ET-1 gene (EDN1). The cell signalling pathways following activation are complex and involve G-protein phosphorylation of multiple pathways including phosphoinositide 3-kinase (PI3K) cascades which trigger calcium channels (Kawanabe Y *et al*, 2002; 2003). ET-1 effects are also mediated partly by protein kinase C (PKC), phospholipase C (PLC) (Sugawara F *et al*, 1996; Bagnato A *et al*, 1997) and phospholipase D (PLD) (Robin P *et al*, 2005). Both of the cyclo-oxygenase -1 and -2 (COX-1 and COX-2) enzymes are involved in tumour progression by promoting tumour angiogenesis, proliferation, survival, invasion and metastasis in several solid tumours. ET-1 has been shown to induce COX-2 and related prostaglandin E_2 release through the activation of the ET_A receptor (Hughes AK *et al*, 1995). There is also a complex interaction between the mitogen activated protein kinase (MAPK) pathway and ET-1 signaling (Song *et al*, 1996).

The pharmacological blocking of the ET_A receptor appears to inhibit tumour progression and there is better understanding of the cellular ramifications of such an action.

A. MC28	Control	ET-1	E/BQ
	1	1.24	1.28
	1	1.23	1.25
	1	1.29	1.24
	1	1.21	1.24
	1	1.19	1.26
	1	1.22	1.24
	1	1.21	1.23
	1	1.23	1.24
Mean	**1**	**1.2275**	**1.2475**
SD		**0.0296**	**0.0158**

B. SW620	Control	ET-1	E/BQ
	1	1.29	1.23
	1	1.35	1.25
	1	1.33	1.26
	1	1.28	1.26
	1	1.36	1.29
	1	1.33	1.38
	1	1.32	1.41
	1	1.33	1.36
Mean	**1**	**1.32375**	**1.305**
SD		**0.0272**	**0.0682**

C. LIM1215	Control	ET-1	E/BQ
	1	1.16	1.21
	1	1.18	1.2
	1	1.18	1.2
	1	1.19	1.19
	1	1.21	1.22
	1	1.22	1.27
	1	1.21	1.26
	1	1.19	1.2
Mean	**1**	**1.1925**	**1.21875**
SD		**0.0198**	**0.0299**

Table 2.1. The effect of ET-1 and the selective ET_B receptor antagonist, BQ-788 (BQ) at a concentration of 10^{-8}M, on cell growth of MC28 (A) (ET-1 used at 10^{-8}M), SW620 (B) (ET-1 at 10^{-7}M), and LIM1215 (C) (ET-1 at 10^{-9}M) all after 48hrs agent exposure. Cell growth was measured using the methylene blue assay and read as absorbance (equivalent to cell number) at 650nm. The absolute absorbance values were then converted to ratios for ease of presentation (experimental / control; control=1) and the means and standard deviation (SD) calculated. Each result is the sum of 8 repeats.

Cells Medians (range)	MC28	SW620	LIM1215
Control	51.7 (+/-3.3)	36.5 (+/-2.4)	42.1 (+/-2.1)
+ ET-1	64.3 (+/-1.3)	43.0(+/-0.6)	69.7 (+/-2.1)
+ ET-1 & A-127722	49.4 (+/-1.1)	37.4 (+/-2.3)	38.3 (+/-3.6)

Table 2.2. Percentage of cells positive for BrDU staining after incubation for 72 hours in DMEM alone (control), ET-1 or ET and ET_A receptor antagonist, A-127722. Cells were visualised by indirect immunohistochemistry using DAB as chromogen and counted manually under the microscope. There was a significant increase in the proportion of MC28 cells binding BrDU on incubation in ET-1 (10^{-8}M) compared to control ($p<0.01$, Mann Whitney). This was reversed on incubation in ET-1 (10^{-8}M) and A-127722 (10^{-11}M) ($p<0.01$). Similarly, for SW620. incubation in ET-1 (10^{-7}M) resulted in a significant increase in the percentage of cells binding BrDU ($p=0.01$) which was reversed on addition of A-127722 (10^{-7}M) ($p=0.015$). LIM1215 also bound more BrDU on incubation in ET-1 (10^{-9}M) ($p<0.01$) and this increase was reversed on incubation in ET-1 and A-127722 (10^{-11}M)($p<0.01$). Experiments were run at least three times in quadriplicates. The Mann Whitney test was applied to the data throughout.

Cells		MC 28		SW 620		LIM 1215	
	C	E	E/A	E	E/A	E	E/A
repeat 1	1	0.76	0.66	0.46	2.5	0.31	1.09
repeat 2	1	0.76	0.69	0.76	2.48	0.46	1.45
repeat 3	1	0.73	0.99	1.33	1.57	0.43	0.43
repeat 4	1	0.78	0.96	0.8	0.21	0.46	0.51
repeat 5	1	0.37	1.12	0.96	0.25	2.09	1.27
repeat 6	1	0.46	0.74	1.13	1.42	1.65	1
repeat 7	1	1.36	1.1	0.86	1.41	0.74	0.78
repeat 8	1	1.4	1.41	0.74	1.09	1.47	1.21
repeat 9	1	1.23	1.43	0.69	0.3	0.96	1.21
repeat 10	1	1.21	0.91	0.37	0.14		
repeat 11				1.15	1.26		
mean		0.906	1.001	0.841	1.148	0.952	0.994
S.D		0.369	0.272	0.290	0.858	0.639	0.351
p-value		>0.05	>0.05	>0.05	>0.05	>0.05	>0.05

Table 2.3. The proportion of apoptotic cells for MC28, SW620 and LIM1215 cells, as visualised by flow cytometry, is displayed. The cells incubated in DMEM only (control, C) were normalised to a value of 1. The proportion of cells exposed to ET-1 (E), and a combination of ET-1 and A-127722 (E/A) were then expressed as a proportion of 1. There were no significant changes in apoptosis in any of the 3 cell lines ($p>0.05$ for all experimental limbs, 1-way ANOVA, Tukeys HSD).

CHAPTER III

Endothelin Receptor Expression in Colorectal Liver Metastases

Aims

This study aims to establish whether or not the ET receptor profile in colorectal liver metastases differs from normal liver tissue and how it compares to previously detected receptor profiles in normal colon and primary colorectal cancer. If there is a detectable change in receptor profile liver metastases may respond differently to ET-1 compared to normal liver cells.

3.1 Introduction

Two main subtypes of ET receptor have been cloned from cDNA libraries, ET_A and ET_B (Sakurai T *et al*, 1992). ET-1 may act as a growth factor for colorectal as well as other cancer cells via one or both of its receptors, ET_A and ET_B.
The development of highly specific agonists and antagonists for these receptors has provided tools for studying the role of ET-1 in tumour vasculature and tumour growth. Radiotracer techniques used in a rat model demonstrated selective tumour vasoconstriction in response to a selective ET_B receptor agonist. Autoradiography applied to the same model showed regions of intense specific ET_B receptor binding scattered throughout the tumour mass (Bell KM *et al*, 1998; 1999). Autoradiography has also detected the presence of binding sites (putative receptors) for ET-1 in colorectal cancer specimens and normal colon tissue (Inagaki H *et al*, 1991 a; 1991b; 1992).

Ali (2000a) demonstrated the up-regulation of ET_A receptors and down-regulation of ET_B receptors in primary human colorectal cancer tissue. Here, we used *in vitro* receptor autoradiography to study the expression of

endothelin receptor subtypes in human colorectal liver metastases and in adjacent normal liver. Micro-autoradiography has also been used in combination with immunohistochemistry to identify binding to specific cell types in slide-mounted tissues.

3.2 Materials and Methods

3.2.1 Tissue collection and preparation

Following approval from the local ethical committee, patients (n=7) undergoing liver resection for colorectal liver metastases were informed and consented for use of the specimens for our research purposes. At the operation, 1 cm^3 samples of liver metastases (n=13) and adjacent normal liver tissue (n=13) (approximately 5cm away) were excised, and immediately cut into small sections, stored in 1ml cryotubes and preserved frozen in liquid nitrogen.

3.2.2 In vitro autoradiography

ET-1 receptors were identified as described previously (Dashwood M *et al*, 1990). Briefly, 10µm frozen sections were cut on a cryostat, then thaw-mounted onto gelatinised microscope slides and air dried. These were either used immediately for experiments or stored at -70°C for future use.

The slide-mounted sections were incubated in 150pM [^{125}I]ET-1(specific activity 2000 Ci/mM) and non-specific binding was determined by co-incubation of alternate slides in the presence of 500nM unlabelled ET-1. ET receptor subtypes were identified using 150pM [^{125}I]-PD151242 for ET_A

receptors or [^{125}I]-BQ3020 for ET_B receptors, specific activity 2000Ci/mM for both (Amersham Pharmacia Biotech, Little Chalfont, Bucks., U.K.).

Autoradiographs generated on 3H Hyperfilm (Amersham Pharmacia Biotech, Little Chalfont, Bucks., U.K.) (figure 1) were semiquantified by densitometric analysis. High-resolution autoradiography was used to identify binding at the cellular level, where slides were coated with a nuclear emulsion (K-2, Ilford, Mobberly, Cheshire, U.K.).

For quantitative assessment images were digitised (Hewlett Packard scanner, Palo Alto, California, USA) and analysed by reading grey densities of the histograms in Adobe Photoshop. In this densitometric analysis, each cm3 was divided into 255 points, each point given a comparative figure from 0 to 250 (0=black, 250=white), equivalent to disintegrations per minute. Means and standard deviations were calculated.

3.2.3 Double labelling

Radioligand binding to endothelial cells was confirmed on selected tissue sections by using a combination of immunohistochemical and autoradiographic techniques. Here, vascular endothelium was identified using PECAM (platelet endothelial cell adhesion molecule-1, CD31). Slides were incubated in primary antibody, anti-CD31 (diluted 1:200 in PBS pH 7.4, for 30 minutes and indirect immunohistochemistry performed using the Vector alkaline phosphatase method (Vector Laboratories, Peterborough, Cambs, UK), with vector red as the chromogen and counterstained with Mayer's haematoxylin. After tissue processing and immunohistochemical staining, slides were transferred to buffer containing [^{125}I]-ET-1, [^{125}I]-PD151242 for

ET_A receptor or [^{125}I]-BQ3020 for ET_B receptor binding as described previously. Sections were coated with nuclear emulsion and stored for 8 days at 4°C in light-proof boxes containing a dessicant. After processing the emulsion following the manufacturer's instructions [^{125}I]-ET-1 binding to endothelial cells was evident as an accumulation of dark grains overlying cells exhibiting positive PECAM immunoreactivity.

3.2.4 Statistical analysis

Analysis of the autoradiography data was carried out using the Wilcoxon signed rank test.

3.3 Results

3.3.1 In vitro Autoradiography

[^{125}I]-ET-1 binding was identified in both normal liver and liver metastases sections. Tissue specimens from liver metastases displayed a significant reduction in ET_B receptor binding ([^{125}I]-BQ3020 binding) in tissue from liver metastases (64.6 x 10^3 gd [grey densities]/mm^2 S.D. 6.9) compared to normal liver tissue (134.5 x 10^3 gd/mm^2 S.D. 16.9) ($p<0.0001$). ET_A receptor density ([^{125}I]-PD151242 binding) was not significantly altered in liver metastases (103.3 x 10^3 gd/mm^2 S.D. 23.2) compared to normal liver tissue (116.7 x 10^3 gd/mm^2 S.D. 15.6) (table 3.1 and figure 3.1).

Micro-autoradiographs, generated on emulsion, showed binding of [125I]-ET-1 to endothelial cells in the tumour tissue (figure 3.2).

3.3.2 Double labelling

[^{125}I]-ET-1 binding to vascular endothelial cells within the liver metastases section was confirmed using CD31 (figure 3.3).

	Liver metastases		Normal liver	
Specimen number	ET_A Mean (SD) gdx1000/mm^2	ET_B Mean (SD) gd1000/mm^2	ET_A Mean (SD) gdx1000/mm^2	ET_B Mean (SD) gdx1000/mm^2
1	94.35 (25.06)	78.55 (14.36)	111.03 (27.84)	126.08 (29.1)
2	88.27 (20.92)	63.84 (11.88)	127.87 (15.8)	154.51 (15.1)
3	85.34 (18.17)	75.85 (18.21)	118.85 (15.21)	151.19 (26.77)
4	91.33 (22.43)	63.67 (25.29)	117.65 (17.32)	111.26 (14.95)
5	90.93 (25.01)	62.52 (21.25)	110.12 (16.17)	111.78 (14.64)
6	124.08 (25.03)	67.86 (16.84)	120.86 (18.4)	138.85 (16.84)
7	123.3 (25.68)	65.8 (8.68)	124.47 (13.52)	133.48 (16.92)
8	76.5 (25.83)	65.21 (15.7)	123.9 (17.99)	159.69 (17.47)
9	76.82 (32.04)	69.73 (18.07)	134 (15.59)	156.41 (15.67)
10	84.19 (23.75)	54.27 (6.11)	144.62 (17.62)	113.28 (19.2)
11	124 (32.81)	56.96 (6.47)	107.12 (23.22)	118.1 (20.82)
12	146.23 (25.57)	57.05 (7.53)	84.62 (19.32)	140.45 (20.45)
13	137.57 (27.92)	58.31 (7.56)	91.81 (21.72)	132.87 (17.19)
Mean	103.3	64.59	116.69	134.46
SD	23.18	6.95	15.58	16.87

Table 3.1. Endothelin receptor expression in specimens of human liver metastases and normal liver, displayed in terms of grey density(gd) /mm^2[equivalent to disintegrations per minute]. The values were derived by semiquantitative densitometry. ET_A receptor density in liver metastases was not significantly changed ($p>0.01$ Wilcoxon signed rank test) from that in normal liver (103.30 S.D. 23.18 vs. 116.69 S.D. 15.58 respectively). ET_B density, however, was significantly ($p<0.01$ Wilcoxon signed rank test) lower in liver metastases than in normal liver (64.59 S.D. 6.95 vs. 134.46 S.D. 16.87 respectively).

The data is displayed in graph form in **Figure 3.1**

Figure 3.1

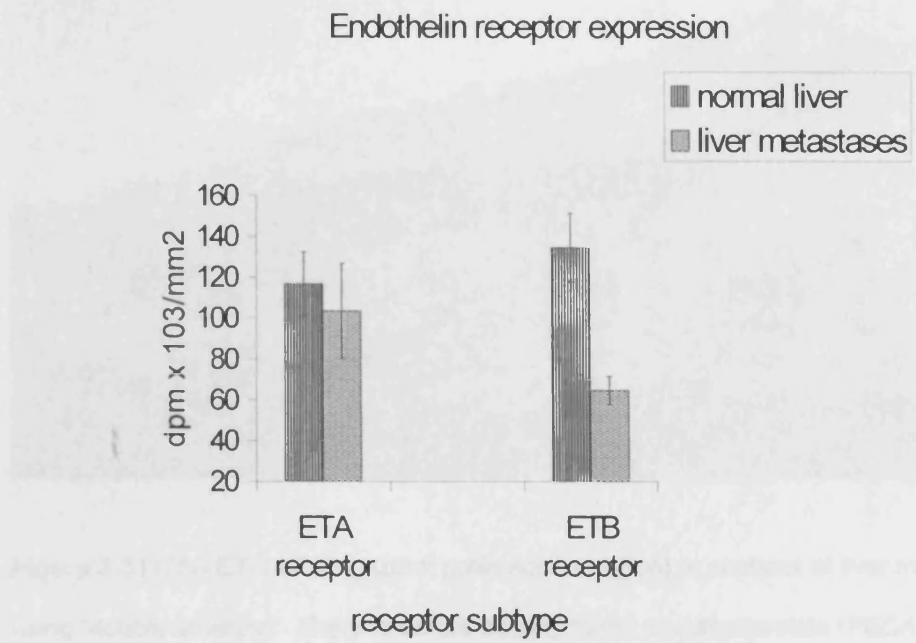
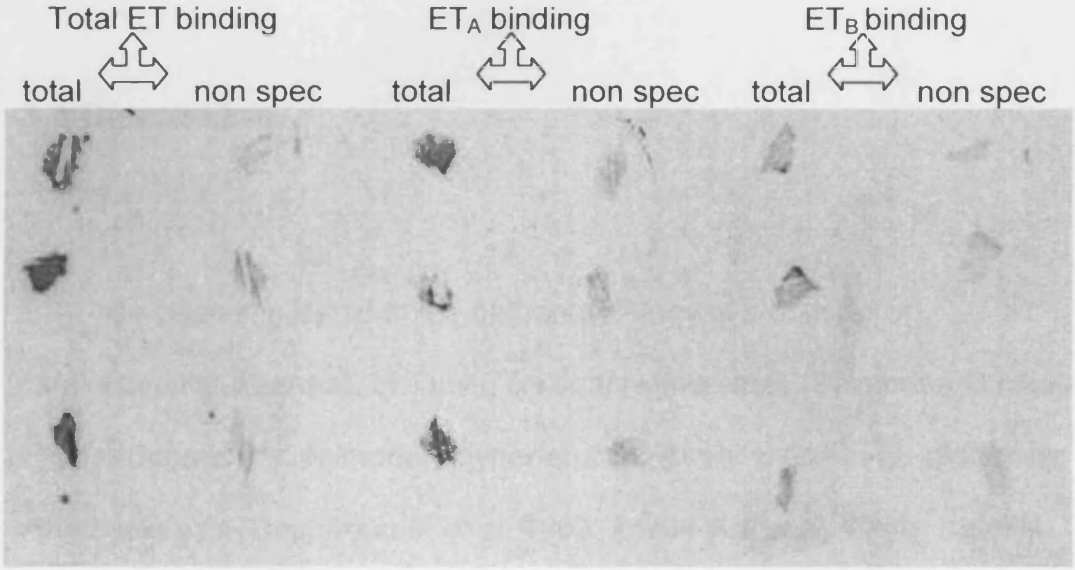


Figure 3.2



ET_A and ET_B receptor binding on sections of liver metastases demonstrated by gross autoradiography. Semiquantitative densitometric analysis was performed on these films. Total ET binding sites are demonstrated on the left followed by ET_A then ET_B binding sites. Non-specific (non spec) binding was excluded from the total.

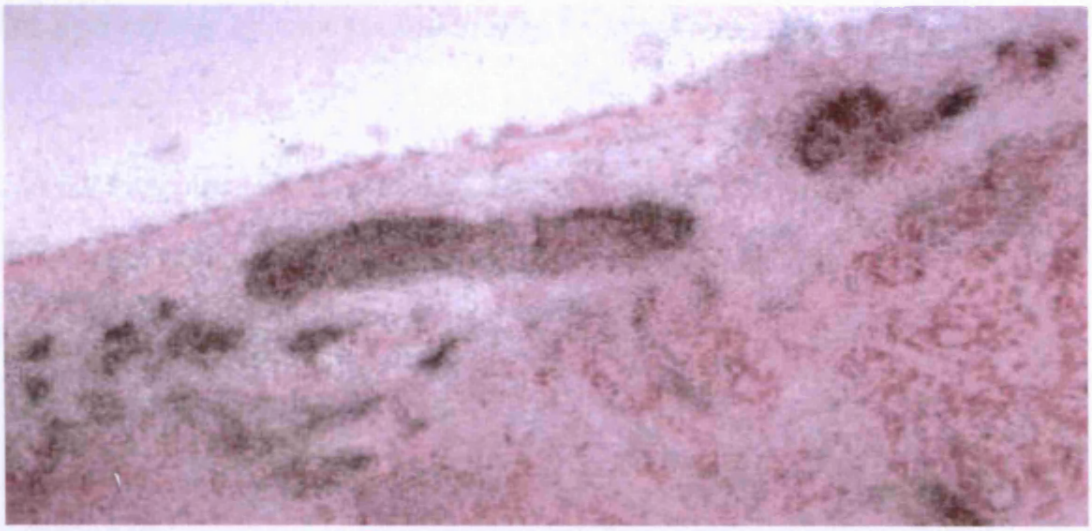


Figure 3.3 [125I]-ET-1 binding (dark grain accumulation) to sections of liver metastases using "double labelling". There is dense binding to the endothelial cells (PECAM-positive, red stain) of tumour vessels.

3.4 Discussion

ET-1 has been implicated in the pathophysiology of a number of cardiovascular diseases, including coronary syndromes (Salomone O *et al*, 1995) systemic and pulmonary hypertension (Rubanyi GM *et al*, 1994) and atherosclerosis (Dashwood M *et al*, 1993; Zeiher AM *et al*, 1994). Studies using autoradiographic binding studies, demonstrated variations in endothelin receptor expression in human coronary vasculature and varicose veins (Dashwood M *et al*, 1998; Agu O *et al*, 2002). Radioligand binding combined with quantitative autoradiography can measure the affinity and localize the distribution of receptors (Inagaki *et al*, 1991a; 1991b; 1992).

Immunocytochemical localization using receptor subtype selective antisera has also been used successfully to map endothelin receptor distribution (Kuc R *et al*, 2004).

Tumour vasculature is morphologically and functionally abnormal (Carmeliet *et al*, 2000). Therefore, the vascular reactivity differs in tumours and there is potential for therapeutic selective modification of tumour blood flow (Brown *et al*, 1998). The vasoactive action of ET-1 is mediated primarily via the two ET-1 receptors. ET_A receptors are mainly found on vascular smooth muscle cells and pericytes. These account for the profound vasoconstriction of ET-1. ET_B receptors are found on contractile elements and endothelial cells and their activation can cause both vasoconstriction and vasodilatation (Davenport AP, 2002).

Additional to vascular pathologies, ET-1 has also been strongly implicated in the pathophysiology of different cancers. It has been shown that ET-1 has mitogenic effects on prostatic (Nelson *et al*, 1996), ovarian (Moraitis *et al*, 1997; Bagnato *et al*, 1999) and colorectal (Ali H *et al*, 2000b) cancer cells in vitro. Shankar (1998) demonstrated that there are elevated levels of ET-1 in the plasma of patients with CRC and colorectal cancer with liver metastases. Working on human pulmonary tumours, Zhao (1995) used a combination of autoradiography and immunocytochemistry to demonstrate that ET-1 binding sites are present on the blood vessels of the tumours. Similar results, using autoradiographic, saturation and competition studies, were obtained by Harland (1998) on human gliomas and meningiomas. In fact the latter group showed that the binding was predominantly to ET_A receptors. ET_A receptor expression is up-regulated in several types of cancer cell lines and in human

colorectal cancer tissue. It has been suggested that the mitogenic stimulus of ET-1 is mediated predominantly via the ET_A receptor (Shichiri M *et al*, 1991; Moraitis S *et al*, 1997). ET-1 stimulates proliferation and migration of endothelial cells through the ET_B receptor (Morbidelli *et al*, 1995; Wren *et al*, 1993; Goligorsky *et al*, 1999) and is a potent mitogen for vascular smooth muscle and tumour cells through the ET_A receptor (Alberts *et al*, 1994; Bagnato *et al*, 1998). Nelson (1996), using autoradiography, and Bagnato (1999), using reverse transcription-PCR and Northern blot analysis, both demonstrated ET-1 production and endothelin receptor expression in prostatic and ovarian cancer cells respectively. In both cases an ET_A receptor antagonist inhibited the ET-1-stimulated growth of cancer cells. Subsequently, Salani and colleagues (2000) localised ET_A receptor expression to ovarian carcinoma cells and blood vessels whereas ET_B receptors were confined to vascular endothelial cells. Autoradiographic binding studies confirmed that ovarian carcinoma cells functionally expressed ET_A , and not ET_B, receptors and that blood vessels co-expressed both receptors (Salani D *et al*, 2000).

Ali (2000a) performed similar work on human CRC cell lines and tissue. This group was able to show, using autoradiography, that both ET_A and ET_B receptors were expressed on the CRC cell lines although ET-1 proliferation signals were transmitted only via ET_A receptors. Furthermore, there was an up-regulation of ET_A receptors and down-regulation of ET_B receptors in human CRC tissue. When these results are considered with those showing over-expression of ET-1 in CRC and colorectal liver metastases tissue (Asham E *et al*, 1997b), there appears a strong case for an autocrine / paracrine role for ET-1, acting via the ET_A receptor, in CRC.

This study further substantiates the work on colorectal cancer tissue (Ali H *et al*, 2000a). ET-1 was identified in both normal liver and liver metastases tissue (unpublished data from our laboratory). Using subtype specific agonists and antagonists, there are demonstrable endothelin receptors in normal and cancerous liver tissue. Therefore, the autocrine / paracrine loop may operate in colorectal liver metastasis. However, there is a reduction in ET_B receptor density in the cancer tissue compared to controls. This is in agreement with findings in prostate cancer (Nelson J *et al*, 1996). However, in our view, since the metastatic tissue is colonic in origin, one must compare the endothelin receptor densitometry results with those obtained in colorectal cancer and in normal colon.

Ali (2000a) demonstrated that the ratio of ET_A:ET_B receptors in normal colon is 0.6:1 (129.2:207.0) and in primary colorectal cancer is 1.7:1 (206.0:122.4). The ratio in colorectal liver metastases is 1.6:1 (103.3:64.6). Thus, there is preservation of the ET_A:ET_B density ratio in metastatic tissue from colorectal cancer. The techniques used for the densitometry were identical in both studies. However, very strong conclusions about the significance of absolute densitometric significance can not be drawn unless one has access to cellular micro-autoradiography. Despite this difficulty, it is interesting that apart from the previously reported apparent over-expression of ET_A receptors in primary CRC, there is a marked under-expression of ET_B receptors both in primary and metastatic CRC tissue. It is possible that ET-1 contributes to the mitogenic drive stimulating CRC cell growth. The ET_A:ET_B ratio increase in both primary and metastatic cancer suggests that an imbalance in this ratio is an event that further drives cancer growth and/or is an established change

"early" in tumourogenesis.

ET_B receptor agonists have been shown to enhance the suppression of endothelial cell apoptosis by ET-1 (Shichiri M *et al*, 1998). We have already shown in chapter 2 that ET-1 produces its stimulatory effect on human CRC cell lines by potentiation of mitosis which is mediated to some degree by ET_A receptors. It is plausible that ET_B receptors also play a role in apoptotic or mitotic changes in CRC. A reduction in ET_B receptor density may reduce apoptosis or increase mitosis.

Interestingly, it is also revealed that ET-1 binding sites are focused at the endothelial cells of the cancer tissue. Therefore, ET_A receptors may play a role in neo-angiogenesis in the tumour, thus aiding mitogenesis further, as suggested by our group (Dawas K *et al*, 1999). However, there is evidence from ischaemic human muscle that ET-1 binding sites on microvessels may well be ET_B receptors (Tsui JC *et al*, 2002).

It has been shown that human primary and metastatic CRC cells display a variation in the expression of endothelin receptors compared to controls. This, combined with the known ET production by CRC *in vivo* and *in vitro*, supports the autocrine / paracrine hypothesis. The ratio of ET_A:ET_B receptors changes from normal colon to colorectal cancer, and this change is preserved in liver metastases. The results discussed here raise the possibility of a therapeutic role for endothelin receptor antagonists in the management of colorectal liver metastases.

The next stage was to test this hypothesis in an animal model.

CHAPTER IV

The effect of an oral ET_A receptor antagonist on colorectal liver metastases in an animal model

Aims

This study aims to assess the effect of an oral ET_A receptor antagonist, A-127722, on colorectal liver metastases in an animal model. The rats were inoculated with syngeneic MC28 rat sarcoma cells. These were previously described as having very similar properties to human colorectal cancer cell lines in vitro, in that their growth curves and dose response to ET-1 and ET receptor antagonists are the same (chapter 2).

4.1 Introduction

Blood vessels within colorectal liver metastases lack innervation and have an incomplete smooth muscle coat (Ashraf S *et al*, 1996). Thus the tumour blood vessels should not possess any significant neurogenic vasoconstrictor capability. This is useful for manipulation of the proportional delivery of chemotherapeutic agents to the tumour by selective vasoconstriction of normal hepatic vessels. Blood would then shunt into the tumour with a concomitant improvement in drug delivery. An animal model of colorectal liver metastases using syngeneic MC28 sarcoma in Hooded Lister rats is appropriate as a model of human liver metastases. It relies on the fact that the rat tumours have a similar blood vessel anatomical and physiological profile to human tumours, including hypovascularity and expression of ET-1 by the same cell types (Loizidou M *et al*, 1991, Loesch A *et al*, 1997; Ashraf S *et al*, 1997). Colorectal liver metastases develop by haematogenous spread through the portal circulation. A pilot study using the syngeneic rat model of liver metastases demonstrated that the intraportal administration of the ET_A specific antagonist, BQ-

123, shortly after tumour cells were inoculated via the same route, resulted in significant reduction in tumour load (Asham E *et al*, 2001). These results may be due to the inhibition of the mitogenic effect of ET-1 but there is strong evidence for the anti-angiogenic effect complimenting the former if not instigating it.

4.2 Methods

4.2.1 Cell Culture

MC28 cells were cultured as outlined in chapter 2. Once they reached 80-90 % confluence they were washed twice in PBS, disaggregated using trypsin/EDTA, then resuspended in Hanks medium to obtain a single cell suspension with a final concentration of 5 x 10^6 cells/ml.

4.2.2 Animals

Inbred male Lister Hooded rats weighing 180-300 g were used. The animals were housed in groups of two or three in standard cages and maintained on a routine rat pellet diet. Rats were divided into two groups: experimental (receiving ET_A antagonist) and control (receiving medium without antagonist). Water (consumption per rat was worked out as roughly 40-45ml per day) was supplemented with the ET_A antagonist, A-127722 (A-127722 (5mg/kg/day) was diluted [1mg in 1ml medium] in medium composed of 50% of 5% dextrose in a solution of 1M Eq. of NaOH, 30% propylene glycol and 20% ethanol), in the experimental limb and with the medium alone in the control limb. The supplementation was started 3 days prior to inoculation of cancer cells and continued for 17 consecutive days.

Twenty four hours prior to the termination of the experiment BrdU was injected into

all rats intra-peritoneally (3mg/100g body weight). All experiments were carried out under a specific project license in accordance with the UK Animal (Scientific Procedures) Act 1986.

4.2.3 Inoculation of Cancer Cells

Anaesthesia was induced in the rats using 3% halothane and maintained on 1.5% halothane and 5L/min oxygen administered via a face mask. A midline laparotomy was performed, the caecum and terminal ileum were delivered through the wound and tributaries of the ileocolic vein were identified in the mesentery. With the aid of an operating microscope 1 x 10^6 MC28 cells (0.2 ml.) were injected into the vein using a 27 gauge needle. Pressure was applied at the puncture site on withdrawal of the needle to avoid backflow of blood. After ensuring satisfactory haemostasis the incision was closed using a continuous 3/0 Prolene® suture.

4.2.4 Tissue Specimens

Terminal anaesthesia was administered to the animals 2 weeks after surgery. The livers were excised, weighed and a note made of the number and size of metastases. The metastases were then dissected out and their total mass measured. The ratio of tumour to total liver mass was calculated (mass of metastases / total mass of liver).

4.3 Results

There were fourteen rats in each limb of the experiment. Three of the rats in the experimental arm and four in the control arm developed mesenteric deposits in the absence of any liver lesions and were excluded. Two of the rats in the experimental

arm and six in the control arm died before the experiment was concluded.

Of the rats that grew liver metastases the T/L ratio in the experimental limb averaged 0.0196 (n=6) whereas in the control limb the ratio was 0.0074 (n=3).

There is no significant difference in the number, size or T/L ratio between the experimental limb and controls (table 4.1).

A-127722	No.	LW	Mets wt	T/L		Control	No.	LW	Mets wt	T/L
1	1	15.03	0.06	0.004		1				
2	25	18.2	1.28	0.07		2	2	11.79	0.05	0.004
3	5	15.14	0.25	0.017		3	11	15.29	0.023	0.0015
4						4				
5						5				
6						6	2	16.87	0.05	0.003
7	1	12.42	0.02	0.002		7				
8						8				
9						9				
10						10				
11	5	14.7	0.18	0.012		11				
12	7	18.6	0.25	0.013		12				
13	4	13.7	0.11	0.008		13	0	19.16	0	
14	18	13.39	1.13	0.084		14	0	17.34	0	

Table 4.1. There were 14 rats in each limb of the experiment (A-127722 is the experimental group and the other is a control group). LW = total liver mass in grams, Mets wt = total mass of liver metastases (grams), T/L= ratio of tumour to total liver mass. No = number of metastases in liver.

4.4 Discussion

The results of this experiment did not yield much useful information. In this section, amongst other topics, I shall discuss the possible reasons for the unsuccessful outcome.

Efficacy studies in animal tumour models provide an early opportunity to collect preliminary information on toxicity (Arp LH, 1999). When screening and evaluating cytotoxic chemotherapeutic agents, efficacy studies usually include at least 1

dosage level that causes severe toxicity and death. Pathologic evaluation in early efficacy studies may reveal major target organs, dosage/schedule relationships, pharmacokinetic/toxicity relationships, effects of formulation and route of administration, maximum tolerated dose, cause of death, and reversibility of changes in normal tissues. Intra-peritoneal formulations are frequently used to establish proof of concept for promising compounds (hits) from *in vitro* screens; however, these crude formulations may also induce intra-peritoneal inflammation and confound the interpretation of both efficacy and toxicity. Efficacy studies conducted in the later stages of drug discovery may be used to refine the dose and schedule proposed for phase I clinical trials. Efficacy studies in animal tumour models provide useful toxicologic data for screening potential drug candidates, optimising the therapeutic index, and designing both preclinical and clinical development programs.

An animal model using two syngeneic rat tumour lines has already shown that the ET_B receptor has an important role to play in modification of tumour blood flow. The ET_B agonist, IRL1620, caused a profound short-term reduction in blood flow in the tumour in the face of an increase in mean arterial blood pressure (Cemazar M *et al,* 2005; Bell *et al*, 1995; 1999). Receptor binding studies also revealed the existence of both ET_A and ET_B receptors in the P22 tumour studied and that ET_A receptors were more abundant than ET_B receptors.

However, in a rat breast tumour model treatment of animals with ET-1 resulted in increased tumour blood flow compared to normal breast tissue in normal rats. This increase was attenuated by pre treatment with the ET_B receptor antagonist BQ-788 (Rai A *et al*, 2003).The pilot study that pre dated this experiment showed a significant inhibitory effect of an ET_A receptor antagonist on the growth of CRC liver

metastases. However, the present larger study does not do the same. There are important differences in the methodologies which will account for this.

A-127722 should be a more potent inhibitor than BQ-123 due to its greater specificity for ET_A receptors. The concentrations used were both within the quoted ones in the original manufacturer's data sheets. However, the mode of deliveries did differ. The pilot study used an intra-portal route for administration whereas the purpose of this study was to investigate the more amenable oral route and to this end the simplest way of designing this was to add the required dose to a fixed amount of water; the assumption is that rats have a predictable intake of water per day according to weight so that there was minimal variation in the concentrations of the agent between the rats. The direct oro-gastric route of administration, by injecting the agent directly via an oro-gastric tube was considered but not used as the rats soon develop an adversity to this route and administration for 17 days would have been very difficult without anaesthesia. Furthermore, the technique was considered cruel.

It is however, impossible to say what the bio-availability of the oral A-127722 in rats is. Furthermore, some rats were more unwell than others after the inoculation of the MC28 cells under general anaesthesia and during this period the water consumption may have varied significantly for a long period of time. If the agent has an increased efficacy at an early stage of the disease, then that window of time would have been missed while the rat was recovering. There were other concerns with our model. The post operative mortality rate was high as was the incidence of mesenteric deposits in the absence of liver metastases. The mesenteric deposits suggest either inadequate intravascular inoculation or preferential anchoring of the MC28 cell lines used in the mesentery. The MC28 cells might have been cross contaminated; higher passage

numbers were less reliable in their inherent production of endogenous ET-1. The medium used for diluting the A-127722 (dextrose / sodium hydroxide / propylene glycol and ethanol) added further uncertainty in that it's effect on the portal circulation is unknown. Several general animal housing issues were also raised around the time of running of this study.

There are other models that have been used and sited. Gervaz (2000) worked on syngeneic BD IX rats and injected DHD K12 colon carcinoma cells into the spleen to produce diffuse liver metastases. Subsequently, TNP-470, a potent angiogenesis inhibitor, was subcutaneously transplanted after tumour implantation on alternate days for 4 weeks. The authors were able to show a significant reduction in the growth of liver metastases. Lauwers (1999), working on 120 WAG/RIJ rats, induced colonic anastomotic tumour and then tested the effect of suramin, an anti angiogenic agent, on intra peritoneal injection. Surprisingly, the tumours grew in size in response to this agent. ET_A receptor intraperitoneal blockade (Atrasentan) in murine tumour human ovarian carcinoma xenografts grown subcutaneously produced a 65% inhibition in tumour growth (Rosano *et al*, 2004). Moreover it potentiated the effect of the chemotherapeutic agent, cisplatinum, significantly.

Anti-angiogenic therapy potentiates radiotherapy in tumour- bearing animals partly because the former reduces hypoxia in the tumour. Furthermore, a combination of anti-angiogenic and cytotoxic therapy has potential for cure in animals with tumours. The effect of the individual agents appears to be merely inhibitory.

Chapter V

Summary and Conclusion of Thesis

Summary of findings:

1. ET-1 stimulates the growth of colorectal cancer and rat sarcoma cells in vitro. The effect had an optimal dose dependent on the cell line investigated.

2. The stimulant effect of ET-1 is mediated via its ET_A receptor. Specific blocking of the ET_A receptor, using A-127722, reversed the ET-1 effect. An ET_B receptor antagonist did not show any effect.

3. ET-1 increase mitosis as measured by BrDU uptake in the cell lines. Apoptosis was not altered by ET-1.

4. The increase in mitosis is thought to be mediated by a G protein subunit Go or Gi and was blocked by pertussis toxin, a specific blocker.

5. The ratio of ET_A:ET_B receptors found in human colorectal liver metastases tissue was increased compared to normal liver tissue taken from the same patients. This correlated positively with findings in colorectal cancer and normal colorectal tissue from previous studies.

6. The oral administration of the ET_A receptor antagonist, A-127722, in an animal model of colorectal cancer liver metastases did not show any significant outcome change. However, the model may have been inadequate. We used the MC28 cell line which is a sarcoma cell line. The numbers of rats that developed the liver metastases were small and the mode of oral delivery of the drug was not reliable enough. A previous pilot study using the same ET_A receptor antagonist

administered through an intraportal parenteral route did show a reduction in tumour load. Other animal models have used, amongst many techniques, subcutaneous tumours, intra splenic tumour injections and intra peritoneal drug administration.

Primary colorectal cancer is surgically curable. However, once there is metastasis the 5-year survival becomes much poorer and the median survival is less than 12 months (Bengtsson G et al, 1981; Lahr CJ et al, 1983). Even the selected patients who benefit from resection only achieve 25-25% 5-year survival (Cady B et al, 1991; Fong et al, 1999; Choti MA et al, 1999). There is potential for improving outcome by reducing the incidence of metastases from colorectal primary tumours. ET-1 is a peptide over-expressed by nearly 100% of colonic cancers. A course of an ET_A antagonist would be combined with resection of the primary tumour. Investigation of the role of ET_A receptor antagonists in vivo may lead to a reduction in or inhibition of cancer cell metastases.

ET-1 antagonism, as a novel form of solid cancer therapy, may compliment conventional therapy. It has several potential advantages over conventional palliative therapy. In general anti-angiogenic therapy targets a small focus of endothelial cells in capillaries at sites of angiogenesis, bone marrow suppression, gastrointestinal symptoms or hair loss are unlikely side effects (Folkman J, 1995). Several issues need addressing in order to take this work forward:

1. The nature of the intracellular signalling following the activation of the G protein.
2. Does ET-1 expression by colorectal cancer and liver metastases correlate with survival?

3. A better model for testing the efficacy of ET_A receptor antagonists in animals.
4. What would be the ideal route of administration of the ET_A receptor antagonist?

The four points outlined above should form the basis of further research into the viability of using ET receptor antagonism as adjuvant treatment for colorectal cancer and liver metastases.

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